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[9] Genetic Fusions as Experimental Tools

By JAMES M. SLAUCH and THOMAS J. SILHAVY

Introduction

Gene fusion technology has revolutionized bacterial genetics. In the past, investigators were limited by the phenotypes and biochemical assays associated with the system under study, and all too often they looked at advances made with *lac*, for example, and wished for equivalent if not similar methodologies. Fusions satisfy this desire because they permit the investigator to adapt a property of choice to the gene of interest.

With the harnessing of transposable genetic elements and the widespread use of recombinant DNA techniques, the available methods for constructing genetic fusions has increased in exponential fashion. It is no longer possible to list all available methods for fusion construction, even if such a compendium is confined to *Escherichia coli*. We have tried, instead, to compile methods for fusion construction that are of interest historically along with those, which seem to us, to be of particular advantage. In addition, we summarize successful strategies employed using fusion strains that we consider of broad general interest. No doubt, our summary is biased and we apologize in advance to our colleagues whose work we have slighted inadvertently.

Fusion Construction

Fusions are constructed by simply creating a novel DNA joint; sequences which were originally separate from one another are made contiguous, such that translational and/or transcriptional signals which affect one, affect the other. The creation of novel joints is most easily accomplished by one of three methods. A commonly used method for the creation of novel joints is recombinant DNA. Table I[1-29] lists a variety of

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plasmid vectors for the creation of fusions. Most of the vectors described are designed for the creation of protein fusions, namely, fusions that result in the formation of a hybrid protein. For the majority of vectors, insertion of the target DNA into the cloning sites, such that the reading frame of the target gene is the same as the reporter gene, results in production of a hybrid protein; the NH_2 terminus is composed of the target protein, and the COOH terminus is the reporter gene. A few of the vectors listed are used for the creation of COOH-terminal fusions, in which the target protein composes the COOH-terminal portion of the hybrid molecule. These constructs are most often used for antibody production. The resulting hybrid is often produced in amounts high enough to form inclusion bodies, which are stable, easily isolated, and contain the hybrid protein in nearly pure form.

A second type of fusion, transcriptional or operon fusions, places an intact reporter gene downstream from the transcriptional start signals of

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TABLE I
PLASMID VECTORS FOR CREATION OF FUSIONS

Vector	Replicon	Marker[a]	Fusion	Type	Sites[b]	Ref.	Comment
pMC1403	ColE1	Amp	LacZ	Protein	RI, S, B	1	Contains *lacZ* without transcriptional or translational

TABLE I
PLASMID VECTORS FOR CREATION OF FUSIONS

Vector	Replicon	Marker[a]	Fusion	Type	Sites[b]	Ref.	Comment
pMC1403	ColE1	Amp	LacZ	Protein	RI, S, B	1	Contains *lacZ* without transcriptional or translational start signals. Sequences are cloned into sites in NH_2 terminus of *lacZ* to create fusion. Natural *Eco*RI site near COOH terminus of *lacZ* has been removed. Fusions are $LacY^+$
pMLB1034	ColE1	Amp	LacZ	Protein	RI, S, B	2	Similar to pMC1403 except vector does not produce LacY
pTSV series	ColE1	Tet	LacZ	Protein	RI, (S, Xh), B	3	Derivative of pMC1403 containing *lacUV5* promoter but lacking translational start site for LacZ. Allows cloning of translational start signals without need to have adjacent promoter. Series includes cloning sites in all reading frames. Fusions are $LacY^+$
pNM480 series	ColE1	Amp	LacZ	Protein	RI, S, B, Sl, Ps, H	4	Derivative of pMC1403 containing multiple cloning site from pUC8. Replicon is pUC8 derived and has higher copy number than pMC1403. Series includes cloning sites in all reading frames. Fusions are $LacY^+$
pLC1	ColE1	Amp, Cm	LacZ	Protein	S, B	5	Derivative of pMLB1034 containing gene for chloramphenicol resistance and strong *rpoL* transcriptional terminator upstream of fusion cloning sites. Fusions are $LacY^-$
pRS414 series	ColE1	Amp	LacZ	Protein	RI, S, B	6	Series of vectors containing strong transcriptional
pRS415 series	ColE1	Amp	*lac*	Operon	RI, S, B	6	terminators 5′ to *lac* sequences, preventing

(*continued*)

TABLE I (*continued*)

Vector	Replicon	Marker[a]	Fusion	Type	Sites[b]	Ref.	Comment
							transcription from upstream promoters; particularly important for operon fusion vectors. Derivatives are available which also contain Kan marker. Cloning sites in all vectors come in either orientation. Series of λ derivatives permit transfer of fusion, including terminator sequences, onto phage vectors by homologous recombination, allowing analysis of fusion in single copy. Plasmid pRS308 allows recovery of fusion from single-copy λ vectors, by homologous recombination. Lac fusions can be switched to Neo fusions using pRS1292 series described below. Fusions are LacY$^+$
pCON5	ColE1	Amp	LacZ	Protein	RI, S, B	7	Derivatives of pMLB1034 which contain M13 *ori* for direct production of single-stranded DNA for sequencing or site-directed mutagenesis. Fusions are LacY$^-$
pCON4	ColE1	Amp	*lac*	Operon	RI, S, B	7	
pKO	ColE1	Amp	*galK*	Operon	RI, H, S	8	Contains intact *galK* gene preceded by cloning sites and translational stop codons in all three reading frames. Used for cloning promoter sequences. Derivative pKG1800 has *gal* promoter cloned into *Eco*RI and *Hin*dIII sites. Used for cloning of transcriptional termination signals. λ derivatives allow transfer of fusion onto phage by homologous recombination for analysis in single copy. Fusions can be recombined into normal *gal* locus from phage derivatives
pPHO7	ColE1	Amp	PhoA	Protein	H, Ps, Sl, X, B, S, K	9	PhoA lacks functional signal sequence. Activity is dependent on contribution from target gene of sequences capable of directing export. *phoA*

Vector	Replicon	Marker	Fusion	Type	Sites	Ref.	Comment
							sequence is bracketed by polylinkers, each containing the restriction sites listed. Cassette-carrying *phoA* can be cloned from pPHOA7 into target gene. *Hin*dIII site is unique at 5′ end of *phoA*. Target gene can be cloned into this site
pJBS633	ColE1	Tet, Kan	Bla	Protein	P (see comment)	10	Vector carries mature portion of β-lactamase from pBR322 with *Pvu*II site that allows insert of foreign DNA. Other unique sites within or upstream of *tet* gene can be used in conjunction with *Pvu*II site for cloning. Use of some of these sites allows expression of fusion protein from *tet* promoter. In-frame fusions which direct export of the hybrid protein confer Amp^r to individual cells. In-frame

							sequencing or site-directed mutagenesis. Fusions are LacY$^-$
pKO	ColE1	Amp	*galK*	Operon	RI, H, S	8	Contains intact *galK* gene preceded by cloning sites and translational stop codons in all three reading frames. Used for cloning promoter sequences. Derivative pKG1800 has *gal* promoter cloned into *Eco*RI and *Hin*dIII sites. Used for cloning of transcriptional termination signals. λ derivatives allow transfer of fusion onto phage by homologous recombination for analysis in single copy. Fusions can be recombined into normal *gal* locus from phage derivatives
pPHO7	ColE1	Amp	PhoA	Protein	H, Ps, Sl, X, B, S, K	9	PhoA lacks functional signal sequence. Activity is dependent on contribution from target gene of sequences capable of directing export. *phoA*

							sequence is bracketed by polylinkers, each containing the restriction sites listed. Cassette-carrying *phoA* can be cloned from pPHOA7 into target gene. *Hin*dIII site is unique at 5′ end of *phoA*. Target gene can be cloned into this site
pJBS633	ColE1	Tet, Kan	Bla	Protein	P (see comment)	10	Vector carries mature portion of β-lactamase from pBR322 with *Pvu*II site that allows insert of foreign DNA. Other unique sites within or upstream of *tet* gene can be used in conjunction with *Pvu*II site for cloning. Use of some of these sites allows expression of fusion protein from *tet* promoter. In-frame fusions which direct export of the hybrid protein confer Amp^r to individual cells. In-frame fusions which do not export hybrid confer Amp^r when cells are patched at high density owing to lysis of some cells in population. Plasmid also contains origin of replication from phage f1 for production of single-stranded DNA for directly sequencing fusion joints
pYZ1	ColE1	Tet	Bla	Protein	RI	11	Vector carries mature portion of β-lactamase from pBR322 with *Eco*RI site that allows insertion of foreign DNA. In-frame fusions which direct export of the hybrid protein confer resistance to Amp to individual cells. In-frame fusions which do not export hybrid will confer resistance to Amp when cells are patched at high density owing to lysis of some cells in population. Plasmid also contains origin of replication from phage f1 for production of

(continued)

TABLE I (*continued*)

Vector	Replicon	Marker[a]	Fusion	Type	Sites[b]	Ref.	Comment
							single-stranded DNA for directly sequencing fusion joints
pYZ4/5	ColE1	Kan	Bla	Protein	See comment	11	Designed for cloning β-lactamase fusions. pYZ4 has *lacUV5* promoter and complementing fragment of *lacZ* with multiple cloning sites. DNA inserts can be identified by loss of α complementation. Plasmid contains f1 origin for production of single-stranded DNA. pYZ5 contains mature portion of β-lactamase with *Pvu*II site on 5′ end and multiple cloning site on 3′ end. Mature β-lactamase cassette can be inserted into pYZ4 clone to form fusions
pRS1292 series	ColE1	Amp	Neo	Protein	RI, S, B, N	12	Analogous to the pRS415 series.[6] Vectors contain transcriptional termination sequences upstream of multiple cloning sites and *neo* gene lacking transcriptional and translational start signals. Derivatives are available with RI, S, B sites reversed. Also, some derivatives contain *lacUV5* promoter/operator in multiple cloning site; introduction into Lac$^+$ cells titrates LacI, causing induction of chromosomal *lac* genes. Recombinant clones which remove *lac* operator from plasmid are identified as no longer inducing *lac*. λ derivatives allow transfer of fusion, including transcription termination signals, to single copy by homologous recombination. In addition, plasmid pRS308 allows recombination of fusion back to high-copy plasmid. Neo fusion can be switched to Lac fusion using pRS415 series described above
pKS11X$_a$	ColE1	Amp	X$_a$–LacZ	Protein	H, X, B, Bg, S, Sl, K	13	Vector for construction of NH$_2$-terminal LacZ fusions linked by short sequence encoding cleavage site for

							protease blood coagulation factor X$_a$. Transcription is directed by *lac* promoter. Fusion protein can be purified and subsequently cleaved with factor X$_a$ to yield NH$_2$-terminal protein fragment. Designed for use in conjunction with λJK2 or pJK2. Can also be used as ORF vector. Fusions are LacY$^-$
pJG200	ColE1	Amp	Collagen–LacZ	Protein	B	14	Vector for construction of NH$_2$-terminal LacZ fusions linked by short sequence from chicken pro-α2-collagen. Transcription is directed by λP$_R$ promoter under control of temperature-sensitive λcI857 repressor, also carried on vector. Fusion protein can be purified and subsequently cleaved with collagenase to yield NH$_2$-terminal protein fragment;

							multiple cloning sites and *neo* gene lacking transcriptional and translational start signals. Derivatives are available with RI, S, B sites reversed. Also, some derivatives contain *lacUV5* promoter/operator in multiple cloning site; introduction into Lac$^+$ cells titrates LacI, causing induction of chromosomal *lac* genes. Recombinant clones which remove *lac* operator from plasmid are identified as no longer inducing *lac*. λ derivatives allow transfer of fusion, including transcription termination signals, to single copy by homologous recombination. In addition, plasmid pRS308 allows recombination of fusion back to high-copy plasmid. Neo fusion can be switched to Lac fusion using pRS415 series described above
pKS11X$_a$	ColE1	Amp	X$_a$–LacZ	Protein	H, X, B, Bg, S, Sl, K	13	Vector for construction of NH$_2$-terminal LacZ fusions linked by short sequence encoding cleavage site for

							protease blood coagulation factor X$_a$. Transcription is directed by *lac* promoter. Fusion protein can be purified and subsequently cleaved with factor X$_a$ to yield NH$_2$-terminal protein fragment. Designed for use in conjunction with λJK2 or pJK2. Can also be used as ORF vector. Fusions are LacY$^-$
pJG200	ColE1	Amp	Collagen–LacZ	Protein	B	14	Vector for construction of NH$_2$-terminal LacZ fusions linked by short sequence from chicken pro-α2-collagen. Transcription is directed by λP$_R$ promoter under control of temperature-sensitive λcI857 repressor, also carried on vector. Fusion protein can be purified and subsequently cleaved with collagenase to yield NH$_2$-terminal protein fragment. Fusions are LacY$^-$
pMR100	ColE1	Amp	cI–ORF–LacZ	Protein	H, B, S, B	15–17	ORF vector. *lac* promoter directs transcription of NH$_2$ terminus of the λ *c*I gene, followed by polycloning site and then out-of-frame *lacI–lacZ* fusion. Insertion of DNA into polycloning site such that frameshift is corrected allows production of cI–ORF–LacZ fusion protein. Fusions are LacY$^-$
pORF1,2	ColE1	Amp	OmpF–ORF–LacZ	Protein	Ps, (Sl, Bg), B, S, B	16, 18, 19	ORF vectors. *ompF* promoter directs transcription of NH$_2$ terminus of *ompF* with polylinker and out-of-frame *lacZ*. Insertion of DNA that restores reading frame produces tribid OmpF–ORF–LacZ protein. pORF1 and pORF2 differ in sites in polylinker and reading frame of *lacZ* versus *ompF*. Fusions are LacY$^-$
pUK270	ColE1	Amp	LacZ–ORF–LacZ	Protein	H, X, Bg, Ps, B, RI	20	ORF vector. *lac* promoter directs transcription of *lac* operon. Polycloning site has been inserted in NH$_2$

(continued)

TABLE I (*continued*)

Vector	Replicon	Marker[a]	Fusion	Type	Sites[b]	Ref.	Comment
							terminus of *lacZ* out of frame. Insertion of DNA that restores frame gives tribid LacZ–ORF–LacZ protein. Fusions are LacY$^+$
pUR series	ColE1	Amp	LacZ–ORF	Protein	B, Sl, (X, P), H	21	Series of vectors for construction of COOH-terminal LacZ fusions. *lac* promoter directs transcription of *lacZ* with multiple cloning sites at 3′ end of *lacZ*. Insertion of open reading frame results in formation of active LacZ–ORF fusion protein. Plasmids differ in reading frame of polycloning sites and in sites themselves. Plasmids with *Pst*I sites have had natural *Pst*I site in *amp* gene destroyed. Fusions are LacY$^-$
pEX series	ColE1	Amp	Cro–LacI–LacZ	Protein	(RI), S, B, Sl, Ps	16, 22	Series of vectors for construction of COOH-terminal LacZ fusions. λ P_R promoter directs transcription of *cro–lacZ* fusion with multiple cloning sites at 3′ end of *lacZ*, followed by translation and transcription termination signals. Insertion of open reading frame results in formation of inactive Cro–LacZ–ORF fusion protein. Plasmids differ in reading frame of polycloning sites and in sites themselves. When induced, fusion protein can account for up to 30% of total cellular protein, often in insoluble and easily isolated form. Fusions are LacY$^-$
pATH series	ColE1	Amp	TrpE–ORF	Protein	See comment	23, 24	Series of vectors for formation of fusions to COOH terminus of TrpE. Vectors differ in cloning sites. Induction with indoleacrylic acid yields high-level synthesis of fusion, which is usually insoluble and stable

λJK2/4	λ	Amp, *imm*^{21}*ts*	*lacZ*	Protein	H, Sp, RI	13	λ vectors for formation of active NH_2-terminal (λJK2) or COOH-terminal (λJK4) fusions to *lacZ*. Phage carry *lac* promoter and gene for Amp resistance. System exists where fusion can be conveniently cloned from phage to plasmid. Fusions are LacY$^-$
λgt11	λ	*imm*$^{\lambda}$*ts*	*lacZ*	Protein	RI	25, 26	λ vector for formation of inactive COOH-terminal fusions to *lacZ* under control of *lac* promoter. Phage carries *Sam* mutation and *c*I857. High-level synthesis of fusion protein can be induced at high temperature without lysis of cells. Fusions are LacY$^-$
λRZ5	λ	—	Lac	Either	—	27	λRZ5 contains the 3′ half of *lacZ* and all of *lacY* adjacent to 3′ half of β-lactamase gene such that

					Ps		LacZ fusions. λ P_R promoter directs transcription of *cro–lacZ* fusion with multiple cloning sites at 3′ end of *lacZ*, followed by translation and transcription termination signals. Insertion of open reading frame results in formation of inactive Cro–LacZ–ORF fusion protein. Plasmids differ in reading frame of polycloning sites and in sites themselves. When induced, fusion protein can account for up to 30% of total cellular protein, often in insoluble and easily isolated form. Fusions are LacY⁻
pATH series	ColE1	Amp	TrpE–ORF	Protein	See comment	23, 24	Series of vectors for formation of fusions to COOH terminus of TrpE. Vectors differ in cloning sites. Induction with indoleacrylic acid yields high-level synthesis of fusion, which is usually insoluble and stable

λJK2/4	λ	Amp, *imm*^{21}*ts*	*lacZ*	Protein	H, Sp, RI	13	λ vectors for formation of active NH_2-terminal (λJK2) or COOH-terminal (λJK4) fusions to *lacZ*. Phage carry *lac* promoter and gene for Amp resistance. System exists where fusion can be conveniently cloned from phage to plasmid. Fusions are LacY⁻
λgt11	λ	*imm*$^{\lambda}$*ts*	*lacZ*	Protein	RI	25, 26	λ vector for formation of inactive COOH-terminal fusions to *lacZ* under control of *lac* promoter. Phage carries *Sam* mutation and cI857. High-level synthesis of fusion protein can be induced at high temperature without lysis of cells. Fusions are LacY⁻
λRZ5	λ	—	Lac	Either	—	27	λRZ5 contains the 3′ half of *lacZ* and all of *lacY* adjacent to 3′ half of β-lactamase gene such that recombination with any pBR322-based Amp-resistant fusion vector in which *bla* and *lac* are transcribed divergently allows transfer of fusion onto phage by homologous recombination. Recombinant phage is Amp resistant and Lac⁺. Phage can then be integrated at *att*λ for analysis
M13mp181/2	M13	—	Lac	Either	—	28	M13 derivatives contain portion of β-lactamase gene and portion of *lacZ* such that recombination with pBR322-based Amp-resistant fusion vector allows transfer of fusion joint onto M13 phage by homologous recombination for sequencing of fusion joint. Recombinant is Amp resistant but Lac⁻ (entire *lacZ* gene is not on phage) Phage contain *bla* gene in opposite orientations

(*continued*)

TABLE I (*continued*)

Vector	Replicon	Marker[a]	Fusion	Type	Sites[b]	Ref.	Comment
λLac Tet	λ	Tet	Lac	Either	—	5	Contains deleted *amp* gene and 3′ end of *lacZ* and *lacY*. Tet resistance gene is in between. Recombination with any pBR322-based Amp-resistant fusion vector where *amp* and *lac* are transcribed in opposite orientations allows transfer of fusion to phage. Recombinant phage is Amp resistant, Lac⁺, and Tet sensitive
pMLB524, pMLB1060, pMLB1094	ColE1	Amp	Lac	Either	See comment	2	pMLB524 is derivative of pMLB1034 containing only 3′ end of *lacZ* beginning at naturally occurring *Eco*RI site. Used to clone previously constructed Lac fusions from any vector in which *Eco*RI site is present in *lacZ*. pMLB1060 is deleted for *lacZ* sequences up to *Sst*I and has addition of a multiple cloning site. pMLB1094 is deleted up to *Cla*I site with a multiple cloning site added. Insertion of appropriate restriction fragment from previously isolated fusions, e.g., carried on specialized transducing phage, results in reactivation of *lacZ*
pSKCAT	R6K	Amp	*cat*	—	—	29	Plasmid contains internal fragment of *phoA* gene followed by promoterless *cat* gene. R6K origin of replication requires function of *pir* gene. In strains lacking *pir* function, plasmid will integrate at PhoA fusions and convert these to transcriptional *cat* fusions. Plasmid contains cis functions for mobilization by broad host range *incP* conjugative functions

[a] Amp, Ampicillin resistance; Cm, chloramphenicol resistance; *imm*, phage immunity; Kan, kanamycin/neomycin resistance; Spc, spectinomycin resistance; Str, streptomycin resistance; Tet, tetracycline resistance.

[b] B, *Bam*HI; H, *Hin*dIII; N, *Nhe*I; RI, *Eco*RI; S, *Sma*I; Sl, *Sal*I; Sp, *Spe*I; X, *Xba*lI; Xh, *Xho*I; Bg, *Bgl*II; K, *Kpn*I; Ps, *Pst*I.

the target gene. In general, vectors designed for the construction of operon fusions by recombinant methods have been problematic because readthrough from plasmid promoters interferes with the monitoring of target promoter activity. As noted in Table I, solutions to this problem involve transcription terminators appropriately placed within the vector to prevent readthrough transcription.

A second method for fusion construction takes advantage of the properties of transposable genetic elements. The minimum requirements for a transposon-based fusion generator are the cis sites required for transposition and the placement, between these sites, of the reporter gene such that there is no interference with translation and/or transcription from outside the transposon. Table II[30–51a] lists many of the transposable elements designed for the creation of fusions to a variety of reporter genes. Unless otherwise stated, the transposons carry their own transposase and are therefore fully competent for transposition. This is sometimes problematic in that fusions created with these vectors are unstable (e.g., Mud). Many of the derivatives listed in Table II are designed to overcome this problem.

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TABLE II
TRANSPOSABLE ELEMENTS

Element	Size (kb)	Marker	Fusion	Type	Ref.	Comment
m-Tn*3*(*lac*)	4.5	Amp	LacZ	Protein	30	System designed for transposition into sequences cloned into pHSS series vectors. Because of Tn*3* immunity, transposition occurs solely into cloned DNA. Transposase is provided in trans. m-Tn*3*(*lac*) transposes from F derivative pOX38::m-Tn*3*(*lac*) to give cointegrate which cannot resolve owing to lack of *res* site. Conjugation into strain lysogenic for λ(p1*cre*) allows resolution at *lox* site carried on transposon. Fusions are $LacY^+$
Tn*5*-*lac*	12	Kan	*lac*	Operon	31	Transposes at 6% of frequency of wild-type Tn*5*. Fusions are $LacY^+$
Tn*phoA*	7.7	Kan	PhoA	Protein	32	*phoA* gene in Tn*phoA* lacks functional signal sequence. Activity is dependent on contribution from target gene of sequences capable of directing export. Can be used to convert LacZ fusions made with Tn*lacZ* to PhoA fusions and vice versa
Tn*lacZ*		Kan	LacZ	Protein	33	Analogous to Tn*phoA*. Can be used to convert PhoA fusions to LacZ fusion and vice versa. Fusions are $LacY^-$
Tn*5*-VB32	5.7	Tet	*neo*	Operon	34	Promoterless *neo* gene from Tn*5* is placed such that insertions next to active promoters gives operon fusions. Transposon also contains Tet resistance gene from Tn*10*
Tn*blaM*	—	Spc	BlaM	Protein	35	Tn*5* derivative with mature portion of *blaM* cloned into IS50L. In-frame fusions to cytoplasmic proteins or cytoplasmic domains of exported proteins confer Amp^r when cells are patched owing to lysis of some cells in population. Fusions which cause export of β-lactamase confer Amp^r to individual cells. Transposon is delivered from either low-copy plasmid with temperature-sensitive replicon or conditionally defective λ phage. Does not work well for insertions into cloned genes
Mini-Tn*10*-LK	4.9	Kan	LacZ	Protein	36	Contains just outermost 63 or 69 bp from IS10R and L, respectively, with truncated *lacZ* and Kan determinant. Transposase is provided in trans from plasmid pNK629. Transposon is carried on conditionally defective λ1205. Fusions are $LacY^-$
TRPLAC fusion hopper	11	Tet	*lac*	Operon	37	Derivative of Tn*10* with *lac* operon replacing all but end of IS10L. Transposon is carried on conditionally defective phage λ1045. Fusions are $LacY^+$
MudI1	37.2	Amp	*lac*	Operon	38	Prototype for Mu-[illegible]

						to convert LacZ fusions made with Tn*lacZ* to PhoA fusions and vice versa
Tn*lacZ*		Kan	LacZ	Protein	33	Analogous to Tn*phoA*. Can be used to convert PhoA fusions to LacZ fusion and vice versa. Fusions are LacY⁻
Tn*5*-VB32	5.7	Tet	*neo*	Operon	34	Promoterless *neo* gene from Tn*5* is placed such that insertions next to active promoters gives operon fusions. Transposon also contains Tet resistance gene from Tn*10*
Tn*blaM*	—	Spc	BlaM	Protein	35	Tn*5* derivative with mature portion of *blaM* cloned into IS50L. In-frame fusions to cytoplasmic proteins or cytoplasmic domains of exported proteins confer Amp^r when cells are patched owing to lysis of some cells in population. Fusions which cause export of β-lactamase confer Amp^r to individual cells. Transposon is delivered

						from either low-copy plasmid with temperature-sensitive replicon or conditionally defective λ phage. Does not work well for insertions into cloned genes
Mini-Tn*10*-LK	4.9	Kan	LacZ	Protein	36	Contains just outermost 63 or 69 bp from IS10R and L, respectively, with truncated *lacZ* and Kan determinant. Transposase is provided in trans from plasmid pNK629. Transposon is carried on conditionally defective λ1205. Fusions are LacY⁻
TRPLAC fusion hopper	11	Tet	*lac*	Operon	37	Derivative of Tn*10* with *lac* operon replacing all but end of IS10L. Transposon is carried on conditionally defective phage λ1045. Fusions are LacY⁺
MudI1	37.2	Amp	*lac*	Operon	38	Prototype for Mu-specialized transducing phage carrying *lac* genes. Constructs are defective for phage production but are transposition competent, i.e., they carry wild-type *A* and *B* genes. Lysogens are temperature sensitive owing to a *c*ts mutation. Fusions are LacY⁺. Mud can be packaged with the use of helper phage
MudI-8	37.2	Amp	*lac*	Operon	39	Derivative of MudI with amber mutations in transposition functions. Transposition occurs in a suppressor plus background or under conditions where Mu*A*,*B* function is provided in trans. Resulting fusions are stable and temperature resistant in suppressor minus background. Fusions are LacY⁺
MudI1678	24	Amp	*lac*	Operon	40	Derivative of MudI1 with internal deletions. Transposition competent and temperature sensitive
MudI1681	15.8	Kan	*lac*	Operon	40	Derivative of MudI1 with deletion of Amp and insertion of *neo* gene from Tn*5*
MudI1734	11.3	Kan	*lac*	Operon	40	Derivative of MudI1681 where transposition functions have been deleted. Functions must be provided in trans. Resulting fusion is stable and temperature resistant

(*continued*)

TABLE II (*continued*)

Element	Size (kb)	Marker	Fusion	Type	Ref.	Comment
MudI5086	14.9	Kan	*lac*	Operon	41	Mini-Mu which contains *neo* and ColE1 origin of replication. Allows *in vivo* cloning of genes with concomitant fusion formation. Fusions are LacY$^+$
MudI5155	15.6	Kan	*lac*	Operon	41	Derivative of MudI5086 that contains *oriT*, the cis site required for RK2 conjugal transfer
MudI5166	15.8	Cm	*lac*	Operon	41	Derivatives of MudI5155 with Cm resistance replacing Kan
Mini-Mu(Tet^r)	17.1	Tet	*lac*	Operon	42	Derivative of MudI1681 with Tet from Tn*10* replacing Kan marker
MudII301	35.6	Amp	LacZ	Protein	43	Analogous to MudI for construction of protein fusions in single step. Phage is transposition competent and temperature sensitive
MudII-8	35.6	Amp	LacZ	Protein	39	MudII301 derivative with amber mutation from Mud1-8. Resulting fusions are stable and temperature resistant. Fusions are LacY$^+$
MudII1678	7.5	Amp	LacZ	Protein	40	Derivative of MudII301 with internal deletions
MudII1681	15.8	Kan	LacZ	Protein	40	Derivative of MudII301 with deletion of Amp and insertion of *neo* gene from Tn*5*
MudII1734	9.7	Kan	LacZ	Protein	40	Derivative of MudII1681 where transpositions functions have been deleted. These functions must be provided in trans. Resulting fusion is stable and temperature resistant
MudII4042	16.7	Cm	LacZ	Protein	44	Derivative of MudII1681 containing Cm and *ori* from pACYC184. Allows *in vivo* cloning with concomitant fusion formation
MudII5085	13.3	Cm	LacZ	Protein	41	Derivative of MudII4042 in which Mu*A,B* transposition genes have been deleted. These functions must be provided in trans. Resulting fusions are stable and temperature resistant
MudII5117	21.7	Kan, Spc-Str	LacZ	Protein	41	Derivative of MudII1678 with low-copy, broad host range IncW pSa-derived origin of replication. Contains genes for spectinomycin and streptomycin resistance
MudII(*lacZU131*,Ap)	35.6	Amp	LacZam	Protein	45	Derivative of MudII301 with LacZ amber mutation U131 (corresponds to amino acid 41 of wild-type LacZ). Allows creation of protein fusions that would normally be detrimental, e.g., fusions to exported proteins. Production of full-length hybrid is dependent on presence of amber suppressor
Mini-Mu*lux*	15	Kan	*lux*	Operon	46	Derivative of MudII1681 that replaces *lac* with the promoterless *lux* operon from *Vibrio fischeri* encoding for two subunits of luciferase and

[redacted]

MudI1-8	35.6	Amp	LacZ	Protein	39	MudII301 derivative with amber mutation from Mud1-8. Resulting fusions are stable and temperature resistant. Fusions are LacY⁺
MudII1678	7.5	Amp	LacZ	Protein	40	Derivative of MudII301 with internal deletions
MudII1681	15.8	Kan	LacZ	Protein	40	Derivative of MudII301 with deletion of Amp and insertion of *neo* gene from Tn*5*
MudII1734	9.7	Kan	LacZ	Protein	40	Derivative of MudII1681 where transpositions functions have been deleted. These functions must be provided in trans. Resulting fusion is stable and temperature resistant
MudII4042	16.7	Cm	LacZ	Protein	44	Derivative of MudII1681 containing Cm and *ori* from pACYC184. Allows *in vivo* cloning with concomitant fusion formation
MudII5085	13.3	Cm	LacZ	Protein	41	Derivative of MudII4042 in which Mu*A*,*B* transposition genes have been deleted. These functions must be provided in trans. Resulting fusions are stable and temperature resistant
MudII5117	21.7	Kan, Spc-Str	LacZ	Protein	41	Derivative of MudII1678 with low-copy, broad host range IncW pSa-derived origin of replication. Contains genes for spectinomycin and streptomycin resistance
MudII(*lacZU131*,Ap)	35.6	Amp	LacZam	Protein	45	Derivative of MudII301 with LacZ amber mutation U131 (corresponds to amino acid 41 of wild-type LacZ). Allows creation of protein fusions that would normally be detrimental, e.g., fusions to exported proteins. Production of full-length hybrid is dependent on presence of amber suppressor
Mini-Mu*lux*	15	Kan	*lux*	Operon	46	Derivative of MudII1681 that replaces *lac* with the promoterless *lux* operon from *Vibrio fischeri* encoding for two subunits of luciferase and enzymes for production of tetradecanol substrate. Light is produced when *lux* operon is inserted downstream from active promoter. Derivative that contains Tet marker in addition to Kan marker also exists
MudIIPR3	4.5	Cm	Neo	Protein	47	Derivative of MudII1734 with promoterless *neo* gene replacing *lacZ*. Transposition functions must be provided in trans
Mud(*lacZ npt*-II)	5.2	—	LacZ, *neo*	Protein/ operon	48	Transposon containing 117 bp at s end and 1006 bp at c end of Mu. Transposition functions must be provided in trans. Creates protein fusion to LacZ and operon fusion to *neo*. Transposon is carried on ColE1 plasmid such that *neo* is not transcribed. Selection for Kan resistance on complementation of Mu*A*,*B* functions selects for transposition events

(*continued*)

TABLE II (*continued*)

Element	Size (kb)	Marker	Fusion	Type	Ref.	Comment
λp*lac*Mu1	~45	*imm*λ	LacZ	Protein	49	Constructed from MudII301 by replacing *amp* and Mu genes with λ genome. Phage is capable of transposition but at reduced frequency owing to deletion of 3′ end of Mu*A* gene.[50] Mu*A*,*B* can be supplied in trans by λpMu507. Resulting fusions are stable. In addition, specialized transducing phage can be isolated that carry fusion and adjacent chromosomal DNA (10 kb). Derivatives exist which are *imm*[21] (λp*lac*Mu3) or that also contain Kan resistance marker (λp*lac*Mu9, Ref. 51). Fusions are $LacY^+$
λp*lac*Mu5	~45	*imm*λ	LacZ	Protein	50	λp*lac*Mu1 derivative with Mu Aam1093. Transposition is completely defective. Derivatives exist which are *imm*[21] (λp*lac*Mu13) or that also contain Kan resistance marker (λp*lac*Mu15)
λp*lac*Mu50	~45	*imm*λ	*lac*	Operon	51	Analogous to λp*lac*Mu1. Replacement of Amp and Mu in MudI1 with λ genome. Transposition frequency is enhanced by supplying Mu*A*,*B* in trans from λpMu507. Derivatives exist which are *imm*[21] (λp*lac*Mu51) or that also contain Kan resistance marker (λp*lac*Mu53). Fusions are $LacY^+$
λp*lac*Mu52	~45	*imm*λ	*lac*	Operon	50	Derivative of λp*lac*Mu50 containing MuAam1093. Completely transposition defective. Derivatives exist which are *imm*[21] (λp*lac*Mu54) or that also contain Kan resistance marker (λp*lac*Mu55)
λp1209	—	*imm*λ	*lac*	Either	2	λ derivative carrying lac operon and c end of Mu. Used for conversion of Mud fusions to λp*lac*Mu fusions by method of Komeda and Iino[51a]

The third method for the creation of novel joints is deletion formation. This can be accomplished by *in vitro* techniques or by selecting for an illegitimate recombination event *in vivo* (the method used initially for the isolation of the fusions).[52] For example, it is not always possible to clone the target gene into a given plasmid vector such that a fusion is created in a single step. A more general *in vitro* approach is to first clone a large portion of the target gene into the vector such that both the target and reporter genes are in the same orientation but not in the same reading frame. In-frame fusions can be subsequently generated by appropriate nuclease treatment and religation. Alternately, the out-of-frame construct can be introduced into a host cell and fusions selected by demanding function and therefore expression of the reporter *in vivo*. These methods are often useful because a large number of fusions containing varying amounts of target gene sequences can be easily isolated.[53]

When choosing a *lac* fusion vector from Tables I or II, several factors, in addition to the comments provided, should be considered. First, many of these vectors do not contain the *lacY* gene, the gene for lactose permease. Without LacY, many of the genetic manipulations which make *lac* fusions so attractive are not available. Although LacZ activity can be monitored with chromogenic substrates or assayed *in vitro* (see below), the host cell is phenotypically Lac$^-$ in the absence of the permease. The presence of *lacY* is indicted for each *lac* vector in the comment sections of Tables I and II.

Many of the early *lac* operon fusion vectors actually express a fusion protein containing the first few amino acids of TrpA fused to LacZ.[54,54a] This is purely for historical reasons; the W209 *trp–lac* fusion, originally constructed *in vivo*, was employed because it provided a convenient means to separate the transcriptional control region of the *lac* operon from the *lacZ* structural gene.[55] Indeed, it was not realized originally that this was actually a protein and not an operon fusion. The Trp sequences, however, do not significantly affect LacZ activity.

Reporter Gene Assays

The chief advantage of gene fusion technology stems from the ability it provides to attach the structural gene for a well-characterized enzyme to any target gene of interest. Thus, with fusion strains, expression of the

[52] S. P. Champe and S. Benzer, *J. Mol. Biol.* **4**, 288 (1962).
[53] M. L. Berman and D. L. Jackson, *J. Bacteriol.* **159**, 750 (1984).
[54] W. W. Metcalf, P. M. Steed, and B. L. Wanner, *J. Bacteriol.* **172**, 3191 (1990).
[54a] J. Zieg and R. Kolter, *Arch. Microbiol.* **153**, 1 (1990).
[55] M. Casadaban, *J. Mol. Biol.* **104**, 541 (1976).

target gene can be simply quantitated by assaying for the reporter. This is a powerful advantage because many important and interesting target gene products are difficult, if not impossible, to assay directly. In a similar vein, gene fusion strains exhibit the characteristic phenotype conferred by the reporter gene. Accordingly, expression of the target gene can be monitored on agar media by scoring activity of the reporter gene product using well-established phenotypic tests. This allows qualitative scoring of large numbers of potentially interesting fusion strains and rapid experimental demonstration of environmental or physiological conditions that alter expression of the target gene. In this section we describe assays and phenotypic tests used for the commonly employed reporter genes.

Spectrophotometric Assays

For several of the commonly used reporter enzymes, commercially available substrates which are colorless in solution yield products that are chromophores with distinctive absorption spectra. These substrates provide the basis for a simple assay that can be accurately monitored with a spectrophotometer.

The compound *o*-nitrophenyl-β-D-galactoside (ONPG) is hydrolyzed by β-galactosidase, yielding *o*-nitrophenol, which is yellow in solution. Miller described an assay for β-galactosidase using this compound that is commonly employed.[56] Indeed, this assay is used so routinely that activity units, which are arbitrary, are referred to in the literature as Miller units. Basically this assay is done with cells permeabilized by chloroform treatment and suspended in an appropriate buffer. After incubation with ONPG for an appropriate time, the reaction is terminated by increasing the pH with Na_2CO_3, and the intensity of yellow color is read in the spectrophotometer. Miller units are then calculated using the formula provided. A fully induced wild-type strain produces about 1000 Miller units of β-galactosidase.

Alkaline phosphatase is assayed using a procedure analogous to that described for β-galactosidase except that *p*-nitrophenyl phosphate (PNPP) is used as substrate. The Beckwith laboratory, which developed this method,[57] uses a formula similar to the Miller formula for LacZ to express activity in arbitrary units.

We[58] have adapted assays for LacZ and PhoA for use with microtiter

[56] J. H. Miller, "Experiments in Molecular Genetics," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972.

[57] E. Brickman and J. Beckwith, *J. Mol. Biol.* **96**, 307 (1975).

[58] J. M. Slauch and T. J. Silhavy, *J. Mol. Biol.* **210**, 281 (1989). [Erratum **212**, 429 (1990).]

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ı, 281 (1989). [Erratum 212, 429 (1990).]

plates and a microplate reader (see also Ref. 59). This procedure requires the purchase of additional equipment, but it offers considerable savings in time if large numbers of assays are to be performed. Software for the IBM PC's is available to perform the necessary calculations using data fed automatically from the microplate reader, and the results are expressed as micromoles of product formed per minute.

Certain cephalosporins such as cephaloridine can be used in a spectrophotometric assay for β-lactamase. In this case, hydrolysis is assayed as a decrease in absorbance at 255 nm.[60] In gram-negative bacteria, however, permeation of this compound across the outer membrane can be problematic. In addition, the behavior of β-lactamase that is internalized in the cytoplasm has not been systematically analyzed. Accordingly, this assay method is seldom used. Instead, bioassays similar to that used for other antibiotic resistance genes are employed (see below).

Assays for Other Reporter Genes

Luciferase activity produced by strains carrying *lux* fusions can be assayed using a scintillation counter in chemiluminescence mode.[46] This assay is extremely sensitive and accurate. However, enzyme activity requires oxygen, reduced flavin mononucleotide ($FMNH_2$), and tetradecanal, and care must be taken to ensure that these do not become rate-limiting.

Galactokinase, the product of the *galK* gene, can be assayed using suspensions of whole cells permeabilized by toluene treatment. The amount of galactose phosphate formed by the phosphorylation of [^{14}C]galactose is measured by filtering the assay mixture through DEAE filters and counting the radioactivity retained on the filter.[8] This assay is sensitive but rather cumbersome in comparison to the chromogenic assays described above.

Typically the relative levels of antibiotic resistance gene expression are determined by bioassay. These tests determine the minimum inhibitory concentration (MIC) of the relevant antibiotic.[10] Alternatively, they identify the concentration of antibiotic required to inhibit the efficiency of plating by 50% (in essense, an LD_{50}).[12] It is important when doing these tests to use cell suspensions of moderate or low cell density; this is especially true for ampicillin-resistant strains.

[59] R. Menzel, *Anal. Biochem.* **181,** 40 (1989).
[60] J. R. Lupski, A. A. Ruiz, and G. M. Godson, *Mol. Gen. Genet.* **195,** 391 (1984).

Specific Activities

In certain cases, particularly when the fusion results in the production of a hybrid protein, activity of the reporter gene product can be markedly different from the cognate wild-type enzyme. Accordingly, it is necessary to correlate the amount of enzyme present with the activity observed. With *lacZ* fusions this can be simply done. β-Galactosidase is larger than most other cellular proteins of *E. coli*, and it migrates on a typical sodium dodecyl sulfate (SDS) gel in a position that is nearly devoid of other proteins. Thus, with a fusion strain exhibiting over 700 Miller units of LacZ activity, the enzyme can be visualized by simply staining the gel with Coomassie blue. With other reporter enzymes, such as PhoA or Bla, or with LacZ when levels are low, immunoprecipitation or Western blots must be used. Knowledge of the specific activity is crucial for some of the uses of gene fusions described below, and this important step is ignored only at great peril.

Phenotypes

The *lac* operon has been extensively studied, and a variety of media have been described that allow the scoring of Lac phenotypes. Four types of media are commonly employed, and recipes for these media are provided in the laboratory manuals of Miller[56] and Silhavy *et al.*[2]

Minimal lactose agar (M63 Lac) can be used with *lacZ* fusion strains that lack the chromosomal *lacZ* gene but are *lacY*$^+$ to determine if β-galactosidase activities are high enough to support growth on lactose as the sole carbon source. Surprisingly low levels of enzyme activity will suffice. The growth rate of strains expressing 50 Miller units is nearly indistinguishable from wild-type.

Lactose MacConkey agar is a rich medium containing bile salts, to inhibit the growth of gram-positive bacteria, and a pH indicator, phenol red. Lac$^-$ *Escherichia coli* grow normally on this medium, but they form white colonies. Lac$^+$ strains form red colonies because lactose fermentation produces acid. This medium is less sensitive than minimal lactose agar for detecting β-galactosidase activity. Strains with low levels of enzyme may grow normally on minimal medium, but form white colonies on MacConkey agar. LacY$^+$ strains expressing about 100 Miller units of β-galactosidase form pink colonies; a wild-type strain forms dark red colonies surrounded by a hazy precipitate of bile salts.

Lactose tetrazolium agar is a rich medium that contains 2,3,5-triphenyl-2*H*-tetrazolium chloride (tetrazolium). Cells growing on this medium reduce the tetrazolium, forming an insoluble, red formazan dye. If the cells ferment lactose, the acid production inhibits formation of the dye. Accord-

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ingly, Lac$^-$ colonies are red, and Lac$^+$ colonies are white. The insoluble nature of the red dye is advantageous because it will not diffuse. This medium is less sensitive than lactose MacConkey agar for detecting β-galactosidase activity. LacY$^+$ strains producing moderate levels of enzyme may form reddish (Lac$^+$) colonies on MacConkey agar yet score as Lac$^-$ (red) on tetrazolium. The sensitivity of lactose tetrazolium agar makes it particularly useful for scoring changes in *lacZ* expression in strains that produce relatively large amounts of β-galactosidase.[61]

In general, LacY activity is required to score β-galactosidase activity on the media described above. However, strains that produce large amounts of β-galactosidase (>1000 Miller units) will grow on minimal agar even in the absence of the permease. For example, *lacI lacY* strains appear Lac$^+$.[62] Phenotypically, LacY activity can be monitored independent of LacZ by scoring growth on melibiose at 42°. At high temperatures, melibiose transport requires LacY.[63]

A very sensitive and commonly used indicator for β-galactosidase activity is the histochemical stain 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). On hydrolysis in the presence of oxygen, this compound yields an insoluble blue dye. Strains producing 1 Miller unit of β-galactosidase can be easily detected. Indeed, many *lacZ* nonsense mutants will form blue colonies after several days owing to low-level misreading. X-Gal works well and can be added to all types of solid media.[2] In addition, it can be used with *lacY* strains.

Each of the media described above (lactose minimal, MacConkey agar, tetrazolium, or media with X-Gal) can be used with *lacZ* fusion strains to score for environmental factors that stimulate *lacZ* expression. For example, fusions of *lacZ* to the *araBAD* operon exhibit higher levels of β-galactosidase in media containing arabinose than in media lacking inducer.[55] However, the medium that exhibits the greatest contrast will depend on the basal (uninduced) level of expression exhibited by the particular fusion. Often X-Gal agar is too sensitive; colonies are always blue, in which case MacConkey or tetrazolium agars should be tried.

Expression of *galK* fusions in strains lacking the chromosomal *galK* gene can be monitored using the minimal, MacConkey, and tetrazolium agars described above by substituting galactose for lactose. In general, the properties of the galactose versions of these media is similar to those employing lactose. In addition, strains lacking galactose epimerase (*galE*) are sensitive to galactose owing to a lethal accumulation of galactose

[61] J. Scaife and J. R. Beckwith, *Cold Spring Harbor Symp. Quant. Biol.* **31,** 403 (1966).
[62] J. Beckwith, personal communication (1990).
[63] J. Beckwith, *Biochim. Biophys. Acta* **76,** 162 (1963).

phosphate.[64] Accordingly, fusions that express galactokinase activity will confer a galactose-sensitive (Gal^{s}) phenotype in *galE* strains.[8] The Gal^{s} phenotype can provide a simple means for scoring environmental conditions that increase the expression of a *galK* fusion.

Expression of *phoA* fusions is most commonly monitored using the compound 5-bromo-4-chloro-3-indolyl phosphate (XP). XP is analogous to X-Gal and is used in similar manner. For best results one should employ a strain lacking the chromosomal *phoA* gene. However, since most media contain phosphate, expression of this gene is repressed, and wild-type colonies appear pale green. If the fusion expresses significant levels of phosphatase activity, the resulting blue colonies are simply detected. Minimal media requiring PhoA activity for growth have also been devised. For example, *β*-glycerol phosphate is not transported by *E. coli,* and cells cannot use this compound as a carbon source unless PhoA is expressed.[65] Alternatively, Tris-based minimal agar with XP as the sole phosphate source can be employed.[65]

The phenotype of strains carrying fusions to antibiotic resistance genes are scored on media containing appropriate concentrations of the relevant antibiotic. These drugs generally work in all solid media. However, we have found that increased sensitivity is observed on MacConkey and tetrazolium agar. Presumably this reflects the damage to envelope structure caused by the various dyes that are present. We routinely add less antibiotic to these media.[2] Also, of course, gene dosage affects the level of resistance directly, and drug concentration should be modified accordingly.

Expression of *lux* fusions can often be monitored simply by examining colonies in the dark.[46]

Genetic and Molecular Manipulations with Fusions

Fusions provide a means to label amino acid sequences with reporter enzyme activity, and they serve to tag target DNA sequences with known sequences from the reporter gene. Accordingly, they can facilitate genetic and molecular analysis of the target gene and its product.

Cloning

Fusions constructed *in vivo* are simple insertion mutations. These insertions provide a marker with which the surrounding DNA can be cloned. Indeed, many of the *in vivo* fusion vectors are particularly suited,

[64] M. B. Yarmolinsky, H. Wiesmeyer, H. M. Kalckar, and E. Jordan, *Proc. Natl. Acad. Sci. U.S.A.* **45**, 1786 (1959).

[65] A. Sarthy, S. Michaelis, and J. Beckwith, *J. Bacteriol.* **145**, 288 (1981).

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acteriol. **145,** 288 (1981).

or even specifically designed, for this purpose, for example, the mini-Mu vectors containing plasmid origins of replication. The use of these vectors is described in detail in [8] in this volume.

The λp*lac*Mu phage are also useful in cloning experiments. As described,[66,67] once the fusion has been isolated, it is a simple procedure to isolate a specialized transducing phage carrying the fusion. Thus, the 5′ end of the gene or operon is cloned. Vectors (Table I) are available for moving these sequences to a plasmid replicon. Alternatively, these sequences can be used for probes to isolate the entire gene by hybridization.

Using Genes to Identify Proteins

Protein fusions expressing a hybrid protein serve to tag the target protein with a domain of known function and antigenic determinants. This provides a convenient method to isolate the target protein sequences. For example, Shuman *et al.*[68] isolated the hybrid protein produced from a *malF′–′lacZ* fusion by virtue of its relatively large size and β-galactosidase activity. The fusion protein was then used to raise antibodies. The antibodies recognize wild-type MalF and allowed characterization of the otherwise unknown protein.

Using Proteins to Identify Genes

It is sometimes the case that a protein has been isolated and characterized but the gene has not been defined. The ORF vectors can be used for cloning any gene for which there are antibodies directed against the corresponding protein. An open reading frame cloned into these vectors allows the production of a functional LacZ fusion. Fusion proteins that react with the particular antibody are identified. The cloned DNA can then be used as probe to isolate the rest of the gene. This approach has been used successfully when only 10 amino acids of the target gene were expressed in the fusion.[69] This type of approach can also be used to delineate specific epitopes of a given protein by cloning various portions of the open reading frames.

Expression in Vivo

Given the current extent to which cloning and DNA sequence analysis has been applied, it is often the case that a putative open reading frame is delineated before the gene is defined genetically. Fusions provide a means

[66] T. J. Silhavy, E. Brickman, P. J. Bassford, M. J. Casadaban, H. A. Shuman, V. Schwartz, L. Guarente, M. Schwartz, and J. Beckwith, *Mol. Gen. Genet.* **174,** 249 (1979).
[67] N. J. Trun and T. J. Silhavy, *Genetics* **116,** 513 (1987).
[68] H. A. Shuman, T. J. Silhavy, and J. R. Beckwith, *J. Biol. Chem.* **255,** 168 (1980).
[69] M. Koenen, U. Ruther, and B. Muller-Hill, *EMBO J.* **1,** 509 (1982).

to determine if the open reading frame is indeed expressed *in vivo*. For example, Kiino *et al.*[70] used this approach to prove that the small open reading frame encoding PrlF was expressed.

Direction of Transcription

Expression of a reporter gene in a fusion requires that the orientation of the reporter be the same as the orientation of the target gene. This provides a means to determine the direction of transcription of the target. For example, fusions constructed with various Mud *lac* phage confer temperature sensitivity to the strain. Isolation of temperature-resistant derivatives often yields deletions. By examining chromosomal deletions that remain Lac$^+$ for loss of adjacent chromosomal markers, the direction of transcription of the target gene can be determined because only markers downstream of the fusion can be deleted such that the fusion remains intact. Wanner *et al.* used this approach to determine the orientation of transcription of a *psi* gene.[71]

The orientation of target genes containing fusions constructed with λp*lac*Mu phage can be studied in a similar fashion. By recombining the *c*I857 mutation onto the phage, the strain can be made temperature sensitive. Selection for temperature resistance selects for deletions. Again, only chromosomal markers downstream of the fusion can be deleted such that the fusion remains intact. Alternatively, the use of λp*lac*Mu allows the isolation of λ specialized transducing phage carrying nearby chromosomal markers.[66,67] By determining the frequency that any given phage carries a nearby marker and the *lac* genes, the relative position of the marker and *lac* can be determined. Phage carrying markers upstream of the fusion will always be Lac$^+$. For markers downstream of the fusion, however, this is not always the case.

The direction of transcription of fusions carried on the chromosome can also be determined by conjugation experiments. For example, F′*lac* can integrate into the chromosome by homologous recombination with *lac* sequences in the fusion, giving rise to an Hfr strain whose direction of transfer is dependent on the orientation of the fusion. By determining the rate of transfer of nearby chromosomal markers, the direction of transfer and hence the direction of transcription of the target gene can be determined.[72]

When fusions are constructed on plasmids, simple restriction analysis

[70] D. R. Kiino, G. J. Phillips, and T. J. Silhavy, *J. Bacteriol.* **172,** 185 (1990).
[71] B. L. Wanner, S. Wieder, and R. McSharry, *J. Bacteriol.* **146,** 93 (1981).
[72] J. Beckwith, E. R. Signer, and W. Epstein, *Cold Spring Harbor Symp. Quant. Biol.* **31,** 393 (1966).

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can reveal the direction of transcription, again, because the orientation of the reporter gene is known.

Transcription Termination

Analysis of transcriptional termination signals at the ends of operons is difficult because of the lack of an apparent phenotype. Fusions can provide the necessary tools for this type of study. Guarente *et al.* used this approach to study the termination signals at the end of the *trp* operon in *E. coli*. A strain containing the promoterless *lacZ* operon downstream of the *trp* terminator is phenotypically Lac$^-$. By selecting for expression of the fusion, mutations which affected termination were isolated.[73]

Gene Regulation

Fusion strains provide many useful tools for the genetic analysis of regulatory systems and the target gene, as evidence by the applications cited below.

Regulons

Genes widely separated on the chromosome, yet controlled by a common regulatory mechanism, are said to be components of a regulon. Because fusions allow rapid screening of physiological conditions that increase or decrease target gene expression, they provide a convenient means to identify regulon components. This principle was first demonstrated by Kenyon and Walker,[74] who screened a collection of random Mud-generated *lacZ* fusion strains for those exhibiting induction with DNA-damaging agents. A similar strategy was employed by Kolodrubetz and Schleif[75] and Wanner and McSharry[76] to identify genes that are induced by arabinose or phosphate starvation. All of these strategies led to the discovery of new genes, and in recent years the method has been used to identify genes whose expression is responsive to a wide variety of environmental conditions.

Autoregulation

Gene fusion technology provides a convenient method for examining target gene expression in the presence of the wild-type target gene product, a condition necessary for studies of autoregulation. Often, specialized

[73] L. Guarente, J. Beckwith, A. M. Wu, and T. Platt, *J. Mol. Biol.* **133,** 189 (1979).
[74] C. J. Kenyon and G. C. Walker, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2819 (1980).
[75] D. Kolodrubetz and R. Schleif, *J. Bacteriol.* **148,** 472 (1981).
[76] B. L. Wanner and R. McSharry, *J. Mol. Biol.* **158,** 347 (1982).

transducing phage carrying the fusion are used to construct the required merodiploids. However, other methods for introducing the fusion in trans can be employed as well. Casadaban,[77] in early work with *araC–lacZ* fusions, employed λ transducing phage to show that AraC is a repressor of its own transcription. In this case, *araC*$^-$ lysogens (the haploid *araC–lacZ* fusion strains) exhibited more β-galactosidase activity than did corresponding *araC*$^+$/*araC–lacZ* merodiploids.

Transcriptional versus Posttranscriptional Control

As described above, fusions can be of two different types. In the first, operon fusions, the reporter gene contains its own translation start signals and is dependent on the target gene for transcription only. In the second, protein fusions, expression of the reporter gene requires both the transcription and translation start signals of the target gene. This difference can be exploited to provide evidence for posttranscriptional control as in the examples cited below.

Expression of the transposase gene, *tnp*, of IS10 is controlled at the level of translation initiation by an antisense RNA.[78] The antisense RNA is produced from a promoter, pOUT, that is located downstream of the *tnp* promoter, pIN, and oriented in the opposite direction. Thus, the antisense RNA overlaps *tnp* mRNA for 38 base pairs (bp) in the region containing the translation start signals. The double-stranded RNA formed cannot be translated, and this helps maintain transposase at low levels. Evidence for this control mechanism was provided by examining the levels of β-galactosidase produced by both *tnp* operon and protein fusions. Levels of β-galactosidase activity produced by the operon fusion are much higher than from the protein fusion. Moreover, β-galactosidase production from the operon fusion cannot be significantly inhibited by high levels of antisense RNA provided in trans. In contrast, β-galactosidase production from the protein fusion is reduced substantially by high levels of the antisense RNA.[78]

Expression of the *gnd* gene of *E. coli* is regulated in response to growth rate, that is, more enzyme is present in fast growing cells. Baker and Wolf[79] found that β-galactosidase production from a series of *gnd–lacZ* operon fusions was not regulated by growth rate, suggesting a posttranslational mechanism. Subsequent work with *gnd–lacZ* protein fusions established translational control since protein fusions are growth-rate regulated. In addition, the studies allowed identification of a site within the *gnd* gene

[77] M. Casadaban, *J. Mol. Biol.* **104,** 557 (1976).
[78] R. W. Simons and N. Kleckner, *Cell (Cambridge, Mass.)* **34,** 683 (1983).
[79] H. V. Baker and R. E. Wolf, *J. Bacteriol.* **153,** 771 (1983).

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71 (1983).

that is required for proper control.[80] Apparently, this internal site functions as a cis-acting antisense RNA to control translation initiation in response to growth rate.[81]

Translational Coupling

There are examples of adjacent genes within an operon where the translational stop signal of the first (promoter proximal) gene overlaps the translational start signal of the second (promoter distal). In such cases, translational coupling is observed, namely, translation of the second gene requires translation of the first. Protein fusions provide a means to detect such coupling. In this case, mutations causing translation termination in the first gene exhibit extreme polarity on fusions to the second. This was demonstrated initially with *trpB* and *trpA*.[82] Other examples of this overlap include *ompR* and *envZ*.[83]

Bifunctional Fusions

Protein fusions with *lacZ, phoA,* and *bla* as the reporter gene produce a hybrid protein with amino acid sequences of the target gene product at the NH_2 terminus and a large, functional, enzymatically active reporter fragment at the COOH terminus. Most of the time, the deletion substitution event that produced the hybrid gene destroys target function. However, this is not always the case. Fusions that retain a large fraction of the target gene sequences may retain function, in which case the hybrid protein is bifunctional, exhibiting both target and reporter gene activities. In addition, many potential target proteins contain distinct functional domains, and the gene fusion may leave a subset of these intact. Such constructs are particularly useful because they provide an active or partially active target protein covalently labeled with reporter enzyme.

Bifunctional Hybrid Proteins

A surprisingly large number of gene fusions exhibit normal, or near normal, activities of both the target and the reporter gene products. The list of proteins that retain function despite the presence of a bulky reporter enzyme covalently attached at the COOH terminus includes cytoplasmic

[80] H. V. Baker and R. E. Wolf, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 7669 (1984).
[81] P. Carter-Muenchau and R. E. Wolf, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1138 (1989).
[82] S. Askov, C. L. Squires, and C. Squires, *J. Bacteriol.* **157,** 363 (1984).
[83] P. Liljestrom, Ph.D. Dissertation, University of Helsinki (1986).

enzymes (TrpA–LacZ[84] and ThrA–LacZ[85]), DNA-binding proteins that regulate transcription (LacI–LacZ[86] and OmpR–LacZ[53]) or function in replication (the replication initiator protein of plasmid R6K–LacZ[87]), and peripheral (MalK–LacZ[88]) and integral cytoplasmic membrane proteins (SecE–PhoA[89]). These novel constructs can facilitate the identification[90] and purification[87] of the target protein. In addition, they can be used to analyze subunit structure[91,92] or interactions with other cellular proteins.[90] A particularly informative example is provided by the MalK–LacZ hybrid protein. MalK is a soluble protein that functions in maltose transport. Using the bifunctional MalK–LacZ hybrid, it was possible to show that it interacts with MalG, an integral cytoplasmic membrane component of the transport system; in *malG*$^-$ strains the hybrid protein (LacZ activity) was soluble, and in *malG*$^+$ strains it was membrane-bound.[90]

Identification of Intragenic Export Signals

Gene fusion technology has been particularly useful for the study of protein export or secretion because many intragenic signals that perform a targeting function are small, discrete, linear sequences of amino acids. Accordingly, these signals behave as independent domains that retain function even in the context of a gene fusion.

PhoA and Bla require periplasmic localization for enzymatic activity, and methods for fusion construction employ 5′ truncations of *phoA* and *bla* that are, in effect, signal sequence deletions (Tables I and II). Thus, fusion strains will exhibit enzymatic activity only if the gene to which these reporters are fused contains a sequence that can function as a signal sequence. This localization-sensitive property has been exploited with Tn*phoA* to screen transposon-induced mutant collections for genes that confer a particular phenotype and specify exported proteins. A good example of the utility of this method is provided by the identification of virulence genes in pathogenic bacteria; many of these gene products are exported (for review, see Ref. 93).

In contrast to PhoA and Bla, LacZ appears to require cytoplasmic

[84] D. Mitchell, W. Reznikoff, and J. Beckwith, *J. Mol. Biol.* **93,** 331 (1975).
[85] I. Saint-Girons, *Mol. Gen. Genet.* **162,** 95 (1978).
[86] B. Muller-Hill and J. Kania, *Nature (London)* **249,** 561 (1974).
[87] J. Germino and D. Bastia, *Cell (Cambridge, Mass.)* **32,** 131 (1983).
[88] S. D. Emr and T. J. Silhavy, *J. Mol. Biol.* **141,** 63 (1980).
[89] P. J. Schatz, P. D. Riggs, A. Jacq, M. J. Fath, and J. Beckwith, *Gen. Dev.* **3,** 1035 (1989).
[90] H. A. Shuman and T. J. Silhavy, *J. Biol. Chem.* **256,** 560 (1981).
[91] G. Heidecker and B. Muller-Hill, *Mol. Gen. Genet.* **155,** 301 (1977).
[92] J. Kania and B. Muller-Hill, *Eur. J. Biochem. (Tokyo)* **79,** 381 (1977).
[93] C. Manoil, J. J. Mekalanos, and J. Beckwith, *J. Bacteriol.* **172,** 515 (1990).

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localization for activity. If, by gene fusion, LacZ is directed to the cellular envelop, enzymatic activity is decreased dramatically, presumably because requisite oligomerization is prevented.[94] In addition, it has been well documented that the cellular export machinery of *E. coli* cannot deal effectively with sequences of LacZ. Thus, if by gene fusion a signal sequence is attached to LacZ and the cell attempts to export the hybrid protein, a lethal jamming of the export machinery can result (for review, see Ref. 95). This phenotype, termed overproduction lethality, has been exploited to obtain signal sequence mutations as outlined below.

Analysis of Membrane Protein Topology

A typical, integral cytoplasmic membrane protein consists of a periplasmic domain separated from a cytoplasmic domain by one or more transmembrane α helices of 20 or so hydrophobic amino acids. The localization-sensitive property of PhoA, Bla, and LacZ fusions can be used to map the disposition of periplasmic and cytoplasmic domains along the primary amino acid sequence of the target gene product. If PhoA or Bla is placed by gene fusion at a position corresponding to a periplasmic domain, then reporter enzyme activity will be high. Conversely, if they are placed in a cytoplasmic domain, activity is low. In contrast, LacZ behaves in an opposite manner: activity is high when the enzyme is cytoplasmic. Thus, gene fusion technology provides a relatively simple means to map membrane protein topology, and while exceptions may be found, these techniques have proved remarkably reliable[10,11] (for PhoA review, see Ref. 93).

Perhaps the most common mistake made with gene fusions when developing a topology map is reliance on activity measurements alone. Low activity can be the result of a variety of uninteresting causes, such as hybrid protein degradation. To be meaningful, activity must be expressed as specific activity, and this, of course, requires an assessment of the amount of hybrid protein present (see above).

Analysis of Protein Folding

In general, reporter gene product activity is insensitive to, and unaffected by, target gene product sequences unless they contain targeting signals that alter or prevent correct cellular localization as mentioned above. However, this is not always true, and some caution is advised. In some cases, the conformation or multimerization of a target domain can

[94] D. Oliver and J. Beckwith, *Cell (Cambridge, Mass.)* **25,** 765 (1981).
[95] K. L. Bieker, G. J. Phillips, and T. J. Silhavy, *J. Bioenerg. Biomembr.* **22,** 291 (1990).

prevent or obscure reporter activity. When this occurs, it can provide a means to analyze amino acid sequences that are crucial for target folding.

A clever example of gene fusion technology applied to the problem of protein folding comes from the work of Luzzago and Cesareni,[96] who analyzed the folding of the H chain of human ferritin, which was produced in *E. coli* using recombinant DNA methodologies. Ferritin is a 24-mer that is, in effect, a molecular cage. In a carefully considered set of experiments, they designed a fusion between ferritin and the α-complementing fragment of LacZ such that the α fragment was sequestered within the ferritin cage. Strains producing the ferritin–LacZα hybrid protein are Lac$^-$ even in the presence of the ω-complementing LacZM15 mutant protein because interaction between the α peptide and the ω fragment is prevented by the ferritin molecular cage. Amino acid substitutions in ferritin can be detected that result in α peptide exposure since they allow intramolecular complementation and a Lac$^+$ phenotype. Some of these changes can be shown to alter the assembly pathway of ferritin.

Mutant Isolation

Fusion technology allows one to tag a particular target gene with a variety of reporter genes, and, as described above, analysis of such strains can provide insights into the regulation, cellular localization, and function of the target gene product. In addition, of course, gene fusions convey to the target gene phenotypic traits of the reporter. Since many target genes of interest confer phenotypes that are difficult and perhaps impossible to select for or against, this can facilitate subsequent genetic analysis substantially. Because the lore of *lac* is vast, and because *lacZ* fusions have been generally available for a longer period of time, they provide an archetype for genetic analysis using gene fusions. Accordingly, the following discussion focuses primarily on *lacZ* fusions. In principle, similar strategies can be carried out with other reporter genes. In fact, in certain situations, other reporters offer distinct advantages, as noted.

Generally speaking, three types of genetic selections or mutant screens can be envisioned for fusion strains. We can look for mutants in which reporter activity is abolished (off), decreased (down), or increased (up). Each of these is discussed in turn. As the examples show, these selections and screens can provide a means to identify genes whose products affect the target in a variety of different ways, from transcription and translation to export or folding.

[96] A. Luzzago and G. Cesareni, *EMBO J.* **8,** 569 (1989).

Selections for Mutation

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Selections for Mutations That Abolish LacZ Activity

Strains carrying a *galE* null mutation are sensitive to galactose owing to the cytoplasmic accumulation of galactose phosphate.[64] Such strains are also sensitive to lactose if the *lac* genes are expressed at reasonable levels, since lactose will be transported and hydrolyzed to yield glucose and galactose.[97] Accordingly, in a *galE–lacZ* fusion strain, selection for resistance to lactose can yield mutants in which *lacZ* expression is abolished. Although this selection works, it has not been extensively employed. Introducing and working with the *galE* mutation in fusion strains is somewhat cumbersome, and we suspect that a high proportion of mutants obtained with this selection will be uninteresting *lacZ, lacY,* or *galK* null mutations. A better strategy may be to employ one of the selections or screens cited in the following section for mutants in which LacZ activity is decreased.

Selections for Mutations That Decrease LacZ Activity

The compound *o*-nitrophenyl-β-D-thiogalactoside (TONPG) is a metabolic poison that is accumulated by *lacY*$^+$ cells to toxic levels. The toxicity of TONPG is best observed in media where cell growth is slow, and relatively high levels of *lacY* expression are required. Provided these conditions are met, it can be used to select mutants in which expression of *lacY* is decreased.

Berman and Beckwith[98] used a *tyrT–lacZ* fusion strain and TONPG to identify mutations that decreased *tyrT* promoter function. In this case, TONPG resistance was selected on minimal succinate agar. To avoid uninteresting mutations such as polar *lacZ* or *lacY* nulls, X-Gal was included in the selective medium. This allowed direct identification of mutants in which expression of both *lacZ* and *lacY* was decreased (TONPG-resistant light blue, not white or dark blue, mutant colonies). A similar strategy was employed by Gutierrez and Raibaud[99] to obtain promoter down mutations affecting the *malPQ* operon. In this case, the selection also yields mutations in *malT*, which specifies the transcriptional activator of the maltose regulon. This demonstrates the utility of the method for identifying positive regulatory genes.

Perhaps the most commonly employed method for identifying mutants that exhibit decreased *lacZ* expression involves the use of lactose indicator media such as MacConkey and tetrazolium agar. Following mutagenesis

[97] M. Malamy, *Cold Spring Harbor Symp. Quant. Biol.* **31,** 189 (1966).
[98] M. Berman and J. Beckwith, *J. Mol. Biol.* **130,** 305 (1979).
[99] C. Gutierrez and O. Raibaud, *J. Mol. Biol.* **177,** 69 (1984).

and plating, mutant colonies that exhibit decreased *lac* expression can be scored. In both types of media, mutants with decreased activity can usually be distinguished from the parent and from mutants in which *lac* expression is abolished. The medium of choice depends on the levels of activity exhibited by the parent strain. For example, when *lac* expression is high in the parent, tetrazolium agar is preferred. Indeed, the first promoter mutations in the *lac* operon were recognized using lactose tetrazolium agar.[61] If *lac* expression in the parent is moderate, MacConkey agar provides a better choice since colonies with moderate expression on tetrazolium agar may appear Lac$^-$ to begin with.

Knowledge of the chromosomal location of regulatory genes permits the use of localized mutagenesis.[2] With this technique, the mutagenic treatment can be focused to a particular chromosomal region that is often distinct from the *lacZ* fusion. Accordingly, every mutant colony scored represents a regulatory mutation. We have used this method extensively to obtain mutational alterations in the positive transcriptional regulatory protein OmpR, for example.[58]

Selections for Mutations That Increase LacZ Activity

It should be clear from the preceding sections that selections for mutants in which *lac* activity is abolished or decreased are fraught with difficulty because of the wide variety of uninteresting mutations that can confer these phenotypes. In general, this is true in all genetic selections for decreased activity. In contrast, selections for increased activity (up) are much more promising since the number of uninteresting mutations that would confer such a phenotype is small. However, in the absence of fusions, this option is often ill-advised or not available. It is not obvious that an up mutation could be obtained in a given operon or regulon by selection for increased activity, because that activity may already be optimum. For example, it is hard to imagine how one would isolate a mutant strain that is more Lac$^+$ or Mal$^+$ than wild type. Fusions alter this situation since environmental conditions that select for increased operon or regulon activity are different from those that select for increased reporter gene activity. Most of the useful genetic methods provided by gene fusions stem from this opportunity to select or screen for mutants in which reporter gene activity is increased.

Strains which contain *malPQ–lacZ* fusions are basically Lac$^-$ in the absence of maltose since operon expression is not induced. Indeed, most of these strains grow poorly on lactose minimal agar. Selection for faster growth yields (in nearly every case) mutants that express the operon constitutively, at least to some degree. In this manner, dominant mutations

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in *malT*, which enable this positive transcriptional activator to function in the absence of maltose (*malT*^{c}), were obtained.[100] Using a similar approach with a strain harboring a *lacZ* fusion to the gene for Tn*3* transposase, Chou *et al.*[101] were able to identify recessive null mutations in a linked gene, *tnpR*, that specifies a transcriptional repressor. These successes demonstrate the utility of gene fusions for identifying regulatory genes and characterizing their mode of action. In both cases, analogous mutations would have been much more difficult to select without the aid of *lacZ* fusions.

The basal level expression of many *lacZ* fusion strains is high enough to permit growth on lactose minimal agar. In this situation, the competitive β-galactosidase inhibitor phenylethylthiogalactoside (TPEG) can be added to the lactose minimal agar to effectively reduce LacZ activity to the point where growth cannot occur. The amount of TPEG required is determined empirically and depends on the particular fusion strain. However, once appropriate conditions are found, mutants that exhibit increased LacZ activity can be selected. Riggs *et al.*,[102] for example, used this method to identify mutations that increase expression of a *secA–lacZ* fusion strain. The report is particularly informative because it also describes two additional methods for identifying mutations that confer LacZ(up) and compares results obtained with the three different methods.

A second method used by Riggs *et al.*[102] utilizes the trisaccharide raffinose. Growth on raffinose requires *lacY*, and, accordingly, the compound can be used to select mutants that exhibit increased *lac* expression. The third method involved EMS (ethyl methanesulfonate) mutagenesis followed by a screen on rich agar containing X-Gal and TPEG. In this case, TPEG addition helped to maximize the color difference between colonies of the parent *secA–lacZ* fusion strain and the LacZ(up) mutants. Riggs *et al.*[102] report 13 LacZ(up) mutants among a population of 3600 mutagenized colonies screened.

It is apparent from the summary provided by Riggs *et al.*[102] that different results are obtained with the different methods. Lactose minimal agar with TPEG yielded more than 1000 mutants with a LacZ(up) phenotype, but a vast majority (96%) were found to be linked to the *secA–lacZ* fusion. Presumably these mutations will help define cis-acting sequences that are involved in *secA* regulation. In contrast, most of the 13 mutants obtained by mutagenesis and colony screening were unlinked to the fusion. Many of these mutations decrease the functional activity of known *sec* gene

[100] M. Debarbouille, H. A. Shuman, T. J. Silhavy, and M. Schwartz, *J. Mol. Biol.* **124**, 359 (1978).
[101] J. Chou, M. J. Casadaban, P. G. Lemaux, and S. N. Cohen, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4020 (1979).
[102] P. D. Riggs, A. I. Derman, and J. Beckwith, *Genetics* **118**, 571 (1988).

products. Such mutants answer this screen because *secA* expression, and thus *secA–lacZ* expression, is derepressed in response to the secretion needs of the cell. In other words, mutations that compromise the export machinery cause *secA* derepression. Among this collection, the authors found a conditional lethal mutation that defined a new *sec* gene, *secE*. The raffinose selection was somewhat disappointing because 288 of the 300 mutant colonies that originally came through the screen did not actually exhibit increased *lacZ* expression. Apparently, these mutations uncovered another permease for raffinose. Among the 12 that exhibited a LacZ(up) phenotype, the distribution of linked to unlinked was intermediate between the other two methods. It is not obvious why the different methods yield different distributions of mutations, but the results underscore the need for investigators to consider alternative approaches.

A final method for obtaining mutants that exhibit a LacZ(up) phenotype utilizes the lactose MacConkey and tetrazolium indicator agars. It is not generally appreciated, but these indicator agars can be used for mutant selection. The lactose concentration in these media is 1%, and, accordingly, mutant colonies with increased *lac* activities have a distinct growth advantage. In addition, since the parent grows, the total number of cells that can be screened on a single plate is enormous. For example, recall that if β-galactosidase is directed to a membrane location by gene fusion, enzyme activity is inhibited (see above). Thus, certain *malE–lacZ* protein fusion strains are basically Lac$^-$. Oliver and Beckwith[94] spread a lawn of such a fusion strain on lactose tetrazolium agar, and after 5 days Lac$^+$ colonies growing out of the parental law could be detected. These colonies are red; for reasons we do not completely understand the color reaction is reversed for colonies on a lawn. Under normal conditions with isolated colonies, a red color indicates Lac$^-$. Mutations identified in this manner include *malE* signal sequence mutations and mutations that decrease the functional activity of components of the cellular protein export machinery. In both cases, a fraction of the LacZ hybrid protein is retained in the cytoplasm, where specific activity is increased substantially. Indeed, the *secA*[94] and *secB*[103] genes were first discovered in this manner.

This section on up mutants would not be complete without reference to the antibiotic resistance genes. When these are used as reporters, up selections are quite simple. One simply increases the antibiotic concentration to the point where the parent fusion strain cannot survive, and under these conditions up mutants can be selected directly.

[103] C. A. Kumamoto and J. Beckwith, *J. Bacteriol.* **154**, 253 (1983).

Novel Phenotypes

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[104] M. Schwartz, M. Roa, ar
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[106] J. Shine and L. Dalgarno,

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Novel Phenotypes

In certain cases, fusion strains can exhibit novel phenotypes that are not conferred by either target or reporter gene mutations. When, and if, this occurs, important new strategies for genetic analysis are provided. Perhaps the most profitable example of this exposure is the overproduction lethality observed with *lacZ* fusions to genes that specify exported proteins.

The cellular export machinery of *E. coli* cannot deal effectively with sequences of β-galactosidase. If, by gene fusion, this machinery is presented with β-galactosidase in large amounts, a lethal jamming occurs, and the cell dies. With *lamB*– or *malE–lacZ* protein fusions, this lethality is evidenced as maltose sensitivity (Mal^s), since maltose addition causes induction of hybrid gene expression (for review, see Ref. 95). The most common mutations that relieve Mal^s are those that prevent hybrid gene expression either by inactivating a regulatory protein or by causing premature translation termination. By devising strategies to avoid these more common lesions, mutations affecting protein export or translation initiation can be uncovered.

Starting with a Mal^s, *lamB–lacZ* fusion strain, Emr and Silhavy[88] selected mutants resistant to maltose. To avoid those that cause expression defects, a Lac$^+$ phenotype was demanded as well. Nearly all of the mutants that answer these criteria contain mutations that alter the LamB signal sequence and prevent export of the hybrid protein. These mutations provided direct evidence that the signal sequence was an important intragenic export signal. Schwartz and colleagues[104,105] employed a similar scheme except that mutations that decreased hybrid protein synthesis were sought; these mutants yielded light blue colonies on agar containing X-Gal. To avoid mutations that cause transcriptional defects, they demanded growth on melibiose (Mel$^+$) at high temperature; under these conditions, melibiose transport is dependent on LacY. Consequently, the phenotype sought was LamB–LacZ(down) LacY$^+$. Mutants which answered this scheme contained mutations that altered the signals required for translation initiation at *lamB*. These mutations provided evidence that mRNA secondary structure in the region near the Shine–Dalgarno[106] sequence can prevent ribosome binding.[104,105]

[104] M. Schwartz, M. Roa, and M. Debarbouille, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2937 (1981).
[105] M. N. Hall, M. Gabay, M. Debarbouille, and M. Schwartz, *Nature* (*London*) **295,** 616 (1982).
[106] J. Shine and L. Dalgarno, *Nature* (*London*) **254,** 34 (1975).

Conclusions

It is not possible to predict with certainty how fusions will be useful in all situations. Based on past experiences fusions will continue to provide opportunities for the geneticist that were heretofore not available. Given that a variety of different fusions can now be simply constructed, their application should always be considered.

[10] Storing, Shipping, and Maintaining Records on Bacterial Strains

By KENNETH E. SANDERSON and DANIEL R. ZEIGLER

Storage Methods

It is extremely important that wild-type and mutant strains be stored by methods which not only assure survival, but which also make certain that the genotype and hence the phenotype of the strains do not change. It is particularly important that the strains be rechecked after they have been stored in permanent culture and before being reported to assure that they still have the properties which are to be described in publication. Storage methods which do not require metabolic activity and cell growth are preferable; methods which allow growth greatly increase the chances of mutation and variation in the cultures.

Freezing

Freezing is the simplest and most common method of storage. For most bacteria, storage in ultracold mechanical freezers (−70° to −90°) is very effective. Storage in −20° commercial freezers is adequate for many bacteria for periods up to 1–2 years but is not recommended for long-term storage. Storage in liquid nitrogen at temperatures from −156° to −196° is superior for cells of some microbial species and for cell lines, but to our knowledge all species of bacteria used extensively for genetic investigation can be maintained at the temperatures achieved by mechanical freezers. Snell,[1] however, reports that the reduced storage temperatures achieved by liquid nitrogen are worth the extra expense.

In order to protect cells from the effects of freezing, cryoprotective

[1] J. J. S. Snell, *in* "Maintenance of Microorganisms" (B. E. Kirsop and J. J. S. Snell, eds.), p. 11. Academic Press, London, 1984.

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[2] R. T. Gerna, *in* "Manual of M
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