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Localized Hydroxylamine Mutagenesis, and Cotransduction of Threonine and Lysine Genes, in *Streptomyces venezuelae*

COLIN STUTTARD

Microbiology Department, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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A lysate of the generalized transducing phage SV1, grown on the prototrophic type strain 10712 of *Streptomyces venezuelae*, was mutagenized with hydroxylamine and used to transduce a lysineless auxotroph to lysine independence on supplemented minimal agar. A complex threonine mutant, strain VS95, was isolated from among the transductants and was shown to be carrying at least two different *thr* mutations. These were about 50% cotransducible with alleles of four independently isolated *lysA* mutations, as were two other independently isolated threonine mutations, *thr-1* and *hom-5*. The location of *thr* genes close to *lysA* occurs in at least three other streptomycetes, but apparently not in *Streptomyces coelicolor* A3(2), in which the *lysA* and *thr* loci are at diametrically opposite locations on the linkage map. This first observation of cotransduction between loci governing the biosynthesis of different amino acids in the genus *Streptomyces* demonstrates the feasibility of fine-structure genetic analysis by transduction in these antibiotic-producing bacteria.

The first reproducible demonstration of generalized transduction in the genus *Streptomyces* was made with phage SV1 in the chloramphenicol-producing species *Streptomyces venezuelae* (7). This phage was originally thought to be intemperate but was subsequently shown to be temperate in several *S. venezuelae* strains (9). Another temperate phage, SF1 in *Streptomyces fradiae* (1), also apparently mediates generalized transduction. Neither of these reports included any observations of cotransducible markers, although recently SV1 was shown to cotransduce three different histidine markers in *S. venezuelae* (manuscript in preparation). Cotransduction of different markers is an essential condition for fine-structure genetic mapping by transduction.

One strategy for the detection of cotransducible genes is to use localized hydroxylamine mutagenesis of transducing phage particles (5). The feasibility of using this technique with SV1 was demonstrated with *S. venezuelae* 3022a (8). However, because auxotrophic mutants of strain 3022a were difficult to isolate, the study of transduction in *S. venezuelae* was shifted to the type strain, 10712, which was much more amenable to mutagenesis.

This paper describes the application of hydroxylamine mutagenesis by transduction and the detection of cotransducible genes involved in aspartate amino acid biosynthesis in *S. venezuelae* 10712.

MATERIALS AND METHODS

Bacteria and phages. All experiments were done with the wild-type and auxotrophic mutants of *S. venezuelae* 10712 (9). Auxotrophs were isolated by replica plating after 8-methoxypsoralen and near UV light mutagenesis of spores to 0.1 to 0.6% CFU survival (10). The frequency of mutants recovered was about 0.5% of CFU plated. Strains used are listed in Table 1. The generalized transducing phage SV1 (7, 9) was used throughout; designations in parentheses indicate the host strain on which each particular lysate was grown.

Culture media and methods. Minimal agar (MM) and supplemented media (designated by MM plus standard biochemical symbols) were as described by Hopwood (6), except that maltose (0.4%, wt/vol) rather than glucose was used as the carbon source. MYM (9) was used as the routine complex medium. Spores were harvested from confluent lawns grown on plates of MYM agar. Phage propagation methods, storage buffer, and transduction procedures were as given previously (7); transducing lysates were routinely irradiated with far UV light (254 nm) to about 0.2% survival (PFU) just before plating, except that no UV light was used after hydroxylamine mutagenesis. Transductants were picked to MYM prespread with rabbit anti-SV1 serum, or sometimes transduction plates were directly replicated to test media spread with anti-phage serum. All incubations were at 30°C.

Hydroxylamine mutagenesis of transducing phage. The method of Hong and Ames (5) was used without modification (8). Briefly, 0.5 ml of SV1 (10712) at 10^{11} PFU/ml in phage buffer (10 mM Tris-hydrochloride [pH 7.5], 4 mM $Ca(NO_3)_2$, 0.005% [wt/vol] gelatin)

TABLE 1. *S. venezuelae* strains used[a]

Strain	Genotype	Phenotype[b]	Source or reference
10712	wt	Prototroph	(9)
VS1	*lysA1*	Lys^-	10712
VS37	*lysA2*	Lys^-	10712
VS52	*lysA3*	Lys^-	10712
VS89	*met-9*	Met^-	*his-5* (derived from 10712) with subsequent reversion to His^+
VS92	*met-10*	Met^-	*his-5* (derived from 10712) with subsequent reversion to His^+
VS90	*met-11*	Met^-	*his-5* (derived from 10712) with subsequent reversion to His^+
VS95	—[c]	Mth^-, feeds VS163	This paper
VS138	*leu-5 cml-6*	Leu^-	*cml-6* (UV-induced mutant of 10712, J. Doull)
VS141	*lysA7 cml-1*	Lys^- Cml^-	*cml-1* (ethyl methane sulfonate-induced mutant of 10712, Z. Ahmed)
VS163	*hom-5 cml-4*	Hom^- or Mth^- Cml^-	UV mutagenesis of *cml-4* (UV-induced from 10712), Z. Ahmed
VS168	*thr-1*	Thr^- Hom^s	Transductant from VS83[d] (×) SV1(10712)
VS175	*thr-1*	Thr^- Hom^s	Revertant of VS83

[a] All mutants were isolated independently after mutagenesis of parental spores with 8-methoxypsoralen plus near UV (10), except as noted.

[b] Abbreviations: Cml^-, chloramphenicol nonproducing; Hom^-, homoserine (or methionine plus threonine) requiring; Hom^s, sensitive to homoserine; Lys^-, lysine requiring, presumptive diaminopimelate decarboxylase deficient; Met^-, methionine requiring; Mth^-, requirement for methionine (or homoserine) plus threonine; Thr^-, threonine requiring; wt, wild type.

[c] Genotype of strain VS95 is complex but seems to include two different threonine mutations, Thr2 and Thr3.

[d] VS83 is a double mutant, *ura-1 thr-1*, derived by two rounds of mutagenesis from strain 10712 via strain VS15 (*ura-1*).

was added to a mixture of 2.5 ml of 0.1 M sodium phosphate buffer (pH 6.0) containing 1 mM EDTA and 2 ml of 1 M hydroxylamine-hydrochloride (Fisher Scientific Co., Fairlawn, N.J.; pH 6.0) which also contained 1 mM EDTA. This mixture was allowed to stand at room temperature for 60 h to give 2 to 10% phage survival (PFU). Phage particles were collected by centrifugation (50,000 × *g* for 90 min), washed once in phage buffer, and suspended in 1 ml of buffer at 4°C to give about 10^{10} PFU/ml. This suspension was then used in transduction experiments without dilution.

Transductions were done on full plates in duplicate with duplicate uninfected recipient control plates and a phage sterility control. Homologous transductions were also done to verify the identity of phage stocks.

Transductant colonies were tested after about 5 to 7 days by using a straight wire to pick and patch spores to MYM prespread with SV1 antiserum. The sample of transductants tested was as near random as possible in that all colonies within one arbitrarily designated sector of each transduction plate were picked (or sometimes all colonies on one whole plate). Patches were allowed to sporulate (usually 3 days) before being replicated to MM and supplemented MM. Each patch plate was also inoculated with prototrophic and donor-type strains as controls.

RESULTS

Isolation of new auxotrophs by transductional mutagenesis. A lysineless mutant (*lysA1*) of strain 10712 was chosen as the recipient in a transduction with hydroxylamine-treated SV1 (10712). This mutant was presumably blocked in the final step of lysine biosynthesis (diaminopimelate decarboxylase) since it grew only on lysine and did not require diaminopimelate (11). Transduction to lysine prototrophy was done on MM supplemented with all amino acids except lysine; six vitamins (biotin, paraaminobenzoic acid, pyridoxine, nicotinic acid, riboflavin, thiamine); and four nitrogenous (DNA) bases (adenine, guanine, cytosine and thymine). The transductant frequency was about 4×10^{-8}/PFU, and 211 colonies were tested for growth on MM, MM + lysine (Lys), and MYM. Six failed to grow on MM + Lys and so apparently carried new auxotrophic mutations. One mutant showed a leaky requirement for homoserine (Hom^-) whereas another (strain VS95) required both threonine and methionine (Mth^-) or threonine and homoserine but failed to grow on homoserine alone or homoserine plus methionine. Therefore, the mutation(s) causing the Mth^- phenotype was probably in one or more threonine genes (see below). The remaining four auxotrophic transductants grew on homoserine plus lysine but were not analyzed any further.

Cotransduction with *lysA*. According to experimental design, the new mutation(s) in strain VS95 should be cotransducible with the original recipient marker, *lysA1*. This was tested by transduction of recipient strain VS95 with donor

TABLE 2. Transductions involving strain VS95

Transduction		Medium[a]	Transductants		
			No. tested	% Phenotype	
Recipient	Donor			Prototroph	Donor[b]
VS95	*lysA1*	Lys	96	37.5	62.5
lysA1	VS95	Met + Thr	50	48	52[c]
			97	31	69[d]
lysA2	VS95	Met + Thr	82[e]	39	61[c]
			146	49	51[f]
lysA3	VS95	Met + Thr	50	38	62[c]
lysA7	VS95	Met + Thr	50	52	48[c]

[a] Supplements added to MM. Lys, Lysine; Met, methionine; Thr, threonine.

[b] Cotransduction frequency = [(number of donor-type transductants)/(number of prototrophs + donor-type transductants)] × 100. It includes putative heterogenotes giving prototrophic progeny (about 5%).

[c] Tested only on MM + Met + Thr.

[d] Includes 15.5% Thr$^-$ Hom^s, 2% Thr$^-$ Hom^i (homoserine insensitive).

[e] Pooled data from two experiments.

[f] Includes 13% Thr$^-$ Hom^s, 2% Thr$^-$ Hom^i.

SV1 (*lysA1*) on MM + Lys. The results (Table 2, top line) show 62.5% cotransduction of *lysA1* and the Mth$^-$ marker(s), and some 5% of the cotransductants were apparently heterogenotes which subsequently segregated prototrophic and donor-type (Lys$^-$) recombinant progeny. One of the Lys$^-$ transductants was verified as SV1 sensitive and was tested to see whether it did indeed carry the *lysA1* mutation; as expected, it failed to give colonies in a cross with SV1 (*lysA1*).

In the reciprocal cross, *lysA1* (×) SV1 (VS95) (Table 2, line 2), the donor marker(s) again appeared (52%) among recombinants on agar supplemented with methionine plus threonine; similar results were obtained with other independently isolated Lys$^-$ mutants (*lysA2*, *A3*, and *A7*; Table 2). However, when the second cross was repeated and transductants were tested for growth on threonine and threonine plus homoserine as well as on methionine plus threonine (Table 2, line 3), two further types of auxotrophic transductants were identified. These were both Thr$^-$, but most colonies were unable to grow in the presence of homoserine (homoserine-sensitive, Hom^s), whereas a minority (about 2% of the total transductants) were insensitive to homoserine (Hom^i). A similar result was obtained in a repeat experiment with the *lysA2* recipient (Table 2, line 5). These data indicate that strain VS95 carried at least two different threonine markers, provisionally designated Thr2 (Thr$^-$ Hom^s) and Thr3 (Thr$^-$ Hom^i), although either marker may be more complex than a single mutation (see below). The low frequency of Thr3 transductants was consistent with a quadruple crossover class from the relation order Thr3-Thr2-*lysA*. Apparently, the combined effect of Thr2 plus Thr3 gave the Mth$^-$ phenotype, but the possibility that strain VS95 carried additional mutations, not detected in this cross, was not excluded.

Cotransduction of different *lysA* and threonine mutation sites. Our stock of mutants of strain 10712 (Table 1) included several threonine or related auxotrophs, two of which, VS163 (*hom-5*) and VS168 or VS175 (*thr-1*), were used in pairwise cotransduction tests with *lysA* mutants. Strain VS163 required homoserine or threonine plus methionine for growth; *thr-1* mutants were very similar to Thr2 strains (Thr$^-$ Hom^s). The results of these transductions (Table 3) confirmed the cotransducibility of *thr* (including *hom*) and *lysA* genes, whereas *met* and *leu* markers were not cotransducible with either *thr-1* or *lysA1*.

Cotransduction of markers within the threonine genes. When VS163 (*hom-5*), VS168 or VS175 (*thr-1*), and VS95 mutants were used in pairwise cotransduction tests, the results (Table 4) were reasonably consistent with the Table 3 data. The low frequency of prototrophs obtained in the cross VS95 (×) *thr-1* was consistent with a very close proximity between *thr-1* and Thr2 sites. The apparent separation of *hom-5* from *thr-1* may indicate that the *lysA* locus lies be-

TABLE 3. Cotransduction of *lysA* and threonine markers

Transduction		Medium[a]	Transductants	
Recipient	Donor		No. tested	Donor type (% cotransduction)[b]
VS168	*lysA1*	Lys	99	27
VS175	*lysA1*	Lys	48	39
lysA1	*thr-1*	Thr	99	46
hom-5	*lysA1*	Lys	66	36
			98	52[c]
lysA1	*hom-5*	Met + Thr	95	52
lysA2	*thr-1*	Thr	98	29
			98	31[c]
VS175	*lysA2*	Lys	100	41
lysA2	*hom-5*	Met + Thr	100	54
lysA3	*thr-1*	Thr	98	37
lysA3	*hom-5*	Met + Thr	99	58.5
lysA7	*thr-1*	Thr	96	47
lysA7	*hom-5*	Met + Thr	95	51.5
met-9[d]	*lysA1*	Lys	100	0
leu-5	*lysA1*	Lys	95	0
leu-5	*thr-1*	Thr	98	0

[a] Supplements added to MM.

[b] See Table 2, footnote *b*.

[c] Results of repeat experiments.

[d] Similar results were obtained with *met-10* and *met-11*.

TABLE 4. Cotransduction of *hom-5* and *thr* mutations

Transduction		Medium[a]	Transductants		
			No. tested	% Phenotype	
Recipient	Donor			Prototrophs	Donor type[b]
hom-5	*thr-1*	Thr	100	51	49
VS95	*thr-1*	Thr	94	3[c]	97[c]
VS95	*hom-5*	Hom	50	52	48[c]

[a] Supplements added to MM. Hom, Homoserine.
[b] See Table 2, footnote *b*.
[c] All mixed colonies (apparent heterogenotes or heterokaryons; see text).

tween the *hom* and *thr* loci, although this order would be expected to give cotransduction frequencies much less than the near 50% observed for *hom-5* and *thr* markers.

DISCUSSION

Successful application of the technique of localized hydroxylamine mutagenesis by transduction in *S. venezuelae* revealed cotransducible genes involved in the biosynthesis of two aspartate amino acids, threonine and lysine. Hong and Ames (5) estimated that treatment of transducing phage P22 with hydroxylamine to about 10% survival gave at least one mutation in 90% of the particles and two in most of them. Therefore, given the size similarity between P22 and SV1 (C. Stuttard and M. Dwyer, unpublished data) and the survival of about 11% PFU in the present experiment, the existence of at least two different *thr* mutations in strain VS95 is not unexpected. The capacity of strain VS95 to grow when supplied with homoserine instead of methionine, in addition to threonine (Table 1), indicates that there is no deficiency in the methionine-specific pathway from the branch-point compound, homoserine, and is consistent with the existence of threonine-specific mutations in this strain. Also, the progeny from transductions involving VS95 included several with complex growth responses to threonine, homoserine, and homocysteine-thiolactone (a methionine precursor) which require further analysis. The relationship between the genotype of strain VS95 and that of the Mth$^-$ mutant, *mthA1*, in *Streptomyces coelicolor* A3(2) (6) is unknown.

The data obtained from a series of two-point ratio transduction tests indicated that *hom-5*, *thr-1*, and mutation sites in strain VS95 are all cotransducible with four sites in *lysA*. The possibility that *lysA* lies between *hom-5* and *thr-1* is not excluded, although the apparent 50% linkage between *hom-5* and *thr-1* (Table 4) as well as with *lysA* sites (Table 3) seems inconsistent with that order. However, the accuracy of data from transductions between strain VS95 and strains VS168 (or 175) (*thr-1*) or VS163 (*hom-5*) (Table 4) is uncertain; similarities in phenotype produced by recipient and donor markers, together with a rather high frequency of mixed transductant colonies and cross-feeding of VS163 by VS95, are possible sources of error in the characterization of transductants. Also, the nutritional requirements of these mutants meant that reciprocal crosses could not be analyzed. The genetic constitution of mixed transductants is presently unknown; they could be heterogenotes resulting from early entry of donor DNA fragments into infected sprouts (germinated spores), or they could be heterokaryons resulting from later entry of donor DNA into sprouts containing more than one genome (6). The reproducibility of the cotransduction frequencies recorded in Table 3 has not been fully tested, but experience with transductions in enterobacteria has shown that even highly consistent two-point ratio data do not always accurately reflect mutation site orders. Nevertheless, the general conclusion that threonine and lysine loci are cotransducible by SV1 in *S. venezuelae* is clearly established. Conjugational mapping has revealed a close proximity between the *thr* and *lys* loci in *Streptomyces rimosus* (4), *Streptomyces glaucescens* (3), and *Streptomyces achromogenes* subsp. *rubradiris* (2). However, it remains to be seen to what extent these similarities reflect an overall conservation of linkage relationships within the streptomycetes.

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