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Transductional Analysis of Chloramphenicol Biosynthesis Genes in *Streptomyces venezuelae*

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Auxotrophs isolated from two chloramphenicol-nonproducing mutants of *Streptomyces venezuelae* included three requiring pyridoxal (Pxl$^-$), VS248 (*cml-11 pdx-2*), VS253 (*cml-11 pdx-3*), and VS258 (*cml-12 pdx-4*), and one requiring thiosulfate, VS263 (*cml-12 cys-28*). Results of SV1-mediated transductions were consistent with the relative marker order *cys-28–cml-12–cml-11–pdx-2,3,4,5*, all of which were cotransducible and must therefore span less than 45 kilobases of DNA, the approximate length of DNA packaged by SV1. *cys-28* was also cotransducible with *arg-4* and *arg-6*, but *arg* and *pdx* were not cotransducible. Results of crosses with donors carrying any one of 11 *cml* mutations were consistent with the location of all *cml* mutations between *cys-28* and *pdx* markers. Also, a new Pxl$^-$ auxotroph (*pdx-6*) and two new Cml$^-$ mutants were recovered after localized hydroxylamine mutagenesis of a *cys-28 cml*$^+$ strain derived from VS263 by transduction.

The biosynthesis of chloramphenicol from chorismic acid follows a relatively simple secondary metabolic pathway which is amenable to genetic analysis. Such analysis should reveal mechanisms for channeling the primary metabolic precursor, chorismic acid, out of the primary pathway to the aromatic amino acids and into the secondary pathway to chloramphenicol.

Our genetic analysis of chloramphenicol biosynthesis in *Streptomyces venezuelae* began with the isolation of 12 mutants blocked in various steps of the pathway (1) (Fig. 1). Two of these mutants (Cml-1 and Cml-12) failed to convert chorismic acid to *p*-aminophenylalanine (PAPA); three (Cml-4, Cml-5, and Cml-8) accumulated PAPA; one (Cml-2) accumulated D-*threo*-1-*p*-nitrophenyl-2-acylamido-1,3-propanediols and was apparently blocked in the chlorination step; and two (Cml-3 and Cml-10) accumulated PAPA intracellularly. The remaining four strains excreted neither chloramphenicol nor any of its aromatic precursors. All 12 mutations were mapped by conjugation to the same arc of the *S. venezuelae* linkage map between *his-6* and *strA6* opposite *adeA10* (2). This paper reports their definition as a single gene cluster by cotransduction with auxotrophic markers (*cys-28* and *pdx*) located by conjugation between *his-6* and *lysA* (2) (Fig. 2). This is the first example of fine-structure analysis of antibiotic biosynthesis genes by generalized transduction.

MATERIALS AND METHODS

Strains used. Auxotrophic derivatives of the 12 Cml$^-$ strains and other mutants used in this study are listed in Table 1. Additional markers were introduced into strain VS258 (*pdx-4 cml-12*) by rather severe nitrosoguanidine mutagenesis (to 1% survival; S. Vats, unpublished data); consequently, multiple mutations were common, as in strain VS335. Strain VS505 (*cys-28 cml*$^+$) was isolated from the transduction VS58 × SV1 (VS263) (see below) done on cystine-supplemented agar.

Preparation of phage. Phage SV1 was propagated on donor strains by the standard agar-layer technique. About 10^6 PFU grown previously in the donor strain were spread on NCG agar [nutrient agar (Difco Laboratories) containing 0.5% glucose and 4 mM $Ca(NO_3)_2$] in two large (140-mm diameter) petri dishes; molten (45°C) soft (0.7%) nutrient agar containing spores of the donor bacteria (ca. 10^6 to 10^7 CFU/ml) was then poured onto each agar surface (ca. 9 ml per plate), and the plates were incubated overnight at 30°C. The soft agar layers containing barely confluent plaques were collected and pooled as a slurry made by adding ca. 12 ml of phage buffer [APB; 10 mM Tris hydrochloride, pH 7.5, 0.005% gelatin, 4 mM Ca $(NO_3)_2$] to each plate and mixing with a glass spreader. The combined slurry was centrifuged for 15 min at ca. 1,000 × *g*; the supernatant fluid was withdrawn, filtered (0.45 μm pore size; Millipore Corp.), and centrifuged at 50,000 × *g* for 90 min at 5°C. The resulting pellet was drained and very gently suspended in 2 ml of APB (usually overnight at 4°C). The lysate was further clarified by a low-speed spin for 15 min (IEC clinical centrifuge at setting 6); it was assayed (usually 2×10^{11} to 5×10^{11} PFU/ml) and stored at 4°C.

Transductions. In half-plate tests, recipient spores (0.05 ml at ca. 10^8 CFU/ml in 20% glycerol) were spread on each transduction plate (usually minimal agar supplemented to select for transductants [5]) and allowed to dry; donor phage (10^{10} PFU/ml) were irradiated with UV light (254 nm) for 3.7 min (to 0.1% survival), and 0.05 ml was spread on one-half of each transduction plate. In full-plate tests, recipient spores and irradiated phage were spread together directly on transduction plates; spores alone and phage alone were spread on corresponding control plates. The nomenclature used for transductional crosses is recipient strain × SV1 (donor strain).

When tight auxotrophic recipients were used, the yield of transductants was enhanced by supplementing the transduction plates with 1% of the usual concentration of nutrient required by the recipient. This level of supplement was sufficient to give barely visible recipient growth similar to the slightly leaky growth shown by most *Streptomyces* auxotrophs. After incubation at 30°C for 3 to 7 days, transductants were picked with a straight wire and patched on agar identical to the transduction medium except for an additional supplement of sodium citrate (10 mM) which prevented further phage infection (4). After sporulation (2 to

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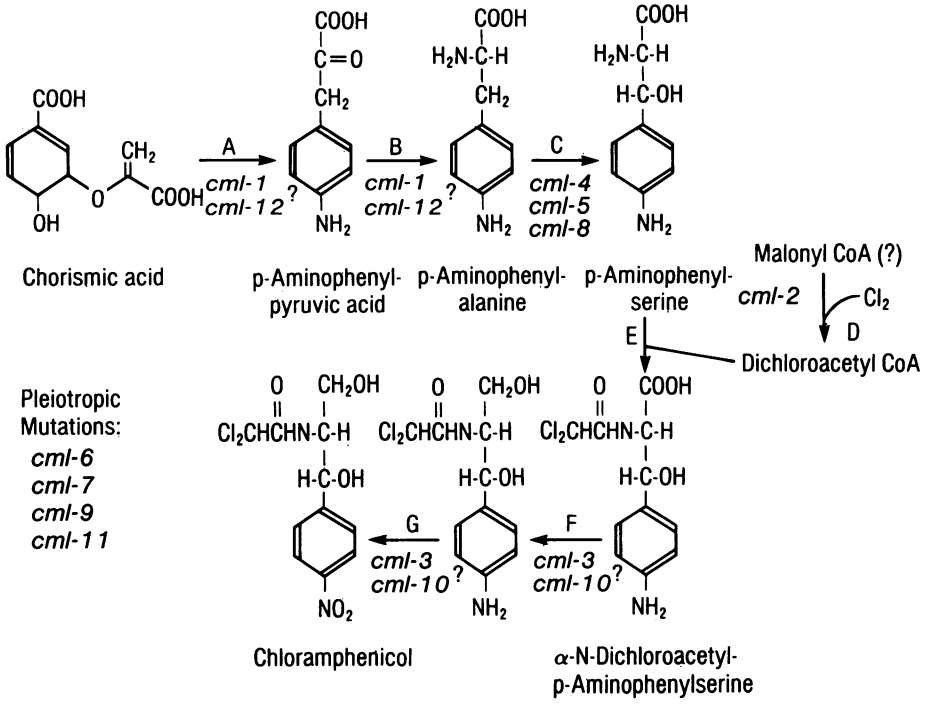

FIG. 1. Location of mutational lesions in the chloramphenicol biosynthetic pathway. Reactions: A, glutaminase (amidotransferase), arylamine synthetase; B, aminotransferase; C, hydroxylase; D, chorination; E, acyltransferase; F, reducatase; G, oxidase. Mutations *cml-1* and *cml-12* impair reaction A or B or both; mutations *cml-3* and *cml-10* may impair reaction F or G. CoA, Coenzyme A.

3 days at 30°C), the patch plates were replicated to diagnostic medium for phenotypic characterization.

Localized mutagenesis. Phage SV1 raised on a wild-type donor was treated with hydroxylamine (5), washed, and used directly in transductions with *cys-28* or *pdx* recipients on medium lacking the nutrient required by the recipient.

Bioassay for chloramphenicol production. The method of Doull et al. (1) was used to assay for chloramphenicol.

RESULTS

Ordering *cml* mutation sites. Initial transduction experiments with phage SV1 (10712) showed high frequencies of cotransduction (72 to 80%) between *cml-11* and *pdx-3* (in VS253) or *pdx-2* (in VS248), while a much lower cotransduction frequency (8%) was observed between *cml-12* and *pdx-4*. The *cml-12* marker was also found to be cotransducible (46%) with *cys-28*. Subsequent crosses (Table 2) showed that *cys-28* was detectably (0.5 to 2%) cotransducible with most *pdx* markers and (about 5%) with *arg* markers, although *arg* and *pdx* were not cotransducible. These results indicated that *cys-28* was situated between *arg* and *pdx* loci and that *cml-12* and possibly *cml-11* were between *cys-28* and *pdx* loci, with *cml-11* being closer to *pdx* while *cml-12* was closer to *cys-28*.

Results of crosses between recipients VS248 (*cml-11 pdx-2*), VS258 (*cml-12 pdx-4*), and VS263 (*cys-28 cml-12*) and donors carrying any one of the other 11 *cml* mutations (Table 3) were consistent with all *cml* mutations being located between *cys-28* and *pdx* markers and gave some indication of the relative order of *cml* markers with respect to these flanking auxotrophic markers. Thus, on the familiar rationale that quadruple crossover recombinants are much less frequent than doubles, the data in line 1 on Table 3 suggest that all Cml$^+$ recombinants arose by two rather than four (or more) crossovers (compare Fig. 3A and B) to give a relative order of mutation sites of *cml-(1* through *10,12)–cml-11–pdx-2* (site order in parentheses is arbitrary), with *cml-11* well separated from all other *cml* mutation sites. Similarly, line 2 data indicate the order *cml-(2,4,7)-12-(1,3,5,6,8-11)–pdx-4* (Fig. 4); while line 3 data (Fig. 5) indicate the order *cys-28–cml-(2,3,4,7)-12-(1,5,6,8-11)*. The composite conclusion drawn from these two-point crosses is given in Fig. 6. The location of *cml-3* is based on the data from Table 3, line 3, which are generally more informative than the data in line 2.

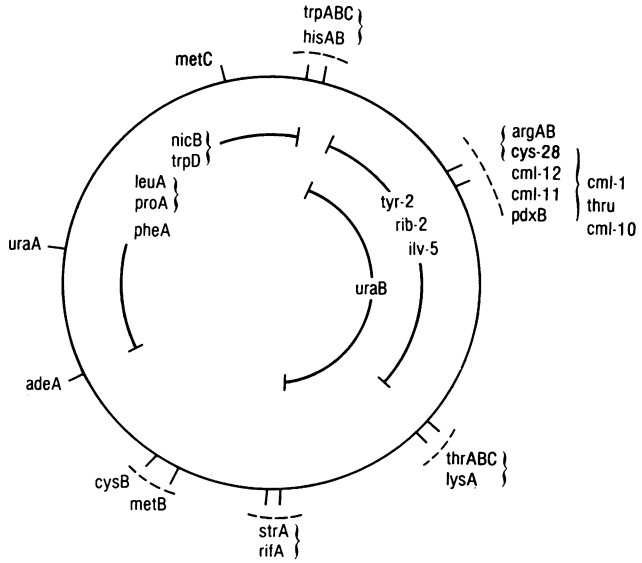

FIG. 2. Chromosomal linkage map of *S. venezuelae*. Bracketed genes are cotransducible by phage SV1.

TABLE 1. Auxotrophic and Cml$^-$ strains of *S. venezuelae* used in this study

Strain	Relevant genotype[a]	Auxotrophic requirement(s)[b]	Derivation		
			Mutagen[c]	Parent strain	Source or reference
VS58	*arg-4*	Arg	MOP	10712[d]	D. Chase
VS98	*arg-6 cml-4*	Orn	MOP	Cml-4	1
VS141	*lysA7 cml-1*	Lys	EMS	Cml-1	1, 5
VS146	*nic-6 cml-7*	Nic	UV	Cml-7	1
VS153	*trp-3 cml-5*	Ind	MOP	Cml-5	1
VS169	*leuB7 cml-3*	Leu	MOP	Cml-3	1
VS211	*his-11 cml-8*	His	MOP	Cml-8	1
VS221	*cys-27 cml-2*	Cys	EMS	Cml-2	1
VS225	*pdx-1 cml-2*	Pxl	EMS	Cml-2	1
VS242	*rib-2 cml-6*	Rib	EMS	Cml-6	1
VS248	*pdx-2 cml-11*	Pxl	EMS	Cml-11	1
VS253	*pdx-3 cml-11*	Pxl	EMS	Cml-11	1
VS258	*pdx-4 cml-12*	Pxl	EMS	Cml-12	1
VS263	*cys-28 cml-12*	Cys	EMS	Cml-12	1
VS273	*pur-15 cml-9*	Ade/Gua	MOP	Cml-9	1
VS276	*leuB13 cml-10*	Leu	MOP	Cml-10	1
VS335	*arg-12 pdx-4 cml-12 cys-45*	Orn Pxl Cys	NG	VS258	This paper
VS373	*arg-16 pdx-4 cml-12*	Orn Pxl	NG	VS258	This paper
VS467	*cys-28 cml-12 pdx-4*	Cys Pxl			This paper
VS499	*pdx-5*	Pxl	EMS	10712	K. Edens
VS500	*pdx-6* (SV1$^+$)	Pxl			This paper
VS505	*cys-28*	Cys			This paper

[a] Standard nomenclature (3). *cml* mutations block chloramphenicol production; VS500 carries prophage SV1; *cys-45* and *arg-12* in VS335 were not cotransducible.

[b] Cys, Cystine or S_2O_3 or S_2O_4 but not S_2O_5; Ind, indole or tryptophan; Orn, ornithine or citrulline or arginine; Pxl, pyridoxal (not pyridoxine); other symbols are standard (3).

[c] EMS, Ethyl methanesulfonate; MOP, 8-methoxypsoralen and UV light (366 nm); NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; UV, UV light, 254-nm wavelength.

[d] The wild type, also known as ISP5230.

TABLE 2. Cotransduction of *arg*, *cys*, and *pdx* markers

Cross[a]		Medium[b]	Transductants	
Recipient	Donor		No. tested	% Donor type[c]
arg-4	*arg-6*	Orn	45	55
arg-4	*arg-12*	Orn	198	47
arg-4	*arg-16*	Orn	158	55
arg-5	*arg-6*	Orn	38	26
arg-4	*cys-28*	Cys + Met	148	5[d]
cys-28	*arg-6*	Orn	288	6[d]
cys-28	*arg-12* (*pdx-4*)	Orn	194	5
arg-16 (*pdx-4*)	*cys-28*	Cys + Pxl	199	1.5
cys-28	*arg-16* (*pdx-4*)	Orn	191	3
cys-28	*pdx-1*	Pxl	97	1
cys-28	*pdx-2*	Pxl	191	0
pdx-2	*cys-28*	Cys + Met	208	2
cys-28	*pdx-3*	Pxl	362	1
pdx-3	*cys-28*	Cys + Met	600	1[d]
cys-28	*pdx-4*	Pxl	197	0.5[e]
pdx-4	*cys-28*	Cys + Met	208	0
cys-28	*pdx-5*	Pxl	196	0[d]
pdx-5	*cys-28*	Cys	350	0
pdx-6	*cys-28*	Cys	396	2[d]

[a] Relevant auxotrophic markers in parental strains; reciprocal crosses were not done with *pdx-1* as recipient (too leaky) or *pdx-6* as donor (VS500 was lysogenic for SV1).

[b] See Table 1, footnote *b*.

[c] Transductants carrying the unselected donor marker. Values represent the nearest percent.

[d] Pooled data, each from two experiments; frequencies of donor-type transductants in crosses with recipient VS58 (*arg-4*) were probably considerably underestimated because VS58 gave a relatively high frequency of prototrophic revertants.

[e] Prototrophs also recovered at frequencies of 0.25 to 1.5% among transductants from the cross VS467 (*cys-28 cml-12 pdx-4*) × VS169 (*cml-3*) on cystine- or pyridoxal-supplemented agar.

However, the high frequencies of Cml$^+$ recombinants from crosses between VS263 and donors with *cml-5*, *cml-6*, *cml-8*, and *cml-9* (Table 3, line 3) by themselves could indicate locations of these donor markers outside the *cys-28–pdx-4* region, but that would not be consistent with the data in line 2, since Cml$^+$ recombinants would then also be expected from these crosses. An additional cross, VS258 × VS225 (*pdx-1 cml-2*), gave 1% Cml$^+$ prototrophs, consistent with the order of *cml-2* and *cml-12* shown in Fig. 6, provided that *pdx-1* lies to the right of *pdx-4* so that only two crossovers

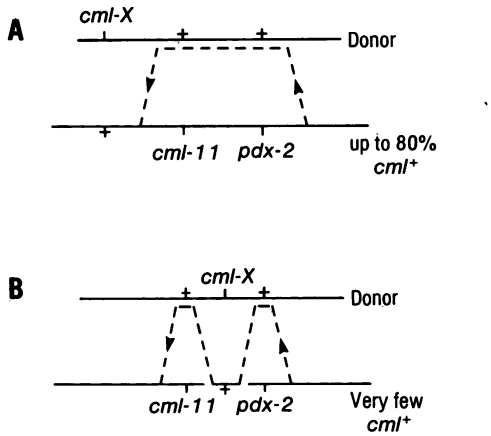

FIG. 3. Alternative arrangements of markers in a transduction between a *cml* donor and recipient strain VS248 (*cml-11 pdx-2*) (see Table 3, line 1). Upper solid lines, fragment of donor DNA; lower solid lines, homologous recipient DNA; dashed lines, recombinant DNA; +, wild-type allele.

TABLE 3. Frequency of Cml$^+$ prototrophic recombinants in transductions between Cml$^-$ donor and recipient strains

Recipient strain	% of ml$^+$ transductants[a] obtained with donor carrying *cml* mutation:												
	1	2	3	4	5	6	7	8	9	10	11	12	+
VS248 (*cml-11 pdx-2*)	72	68	72	87	87	76	69	82	85	76		69	80[b]
VS258 (*cml-12 pdx-4*)	0	2	0	5	0	0	3	0	0	0	0		8
VS263 (*cys-28 cml-12*)	36	2	1	11	48	56	1	50	50	39	30		46

[a] Approximately 200 colonies tested from each cross. Values represent the nearest percent.
[b] A similar result was obtained with VS253 as the recipient.

would be required to generate Cml$^+$ prototrophs. Similarly, the cross VS248 × VS225 gave 40% Cml$^+$ prototrophs, consistent with the mutation site order *cml-2,11–pdx-2,1*; Cross VS500 × VS253 gave very few prototrophs, five of which were tested; all were Cml$^+$, indicating the order *cml-11–pdx-3,6*. Other pairwise crosses between *pdx*-marked strains each gave prototrophic transductants, indicating an absence of any overlapping mutation sites and apparently very close proximity between *pdx-2* and *pdx-3* (data not shown).

Localized mutagenesis. Since all of the auxotrophic mutations flanking the *cml* genes were already coupled with *cml* mutations, it was necessary to construct a Cml$^+$ derivative of one of these for use in the isolation of new *cml* markers. Therefore, strain VS505 (*cys-28 cml*$^+$) was recovered from the cross VS58 × SV1 (VS263) on cystine-supplemented agar. When phage SV1 was treated with hydroxylamine and then immediately used for transduction with recipient strain VS505 on agar supplemented with pyridoxal, arginine, aromatic amino acids, and *p*-aminobenzoic acid, the resulting transductants included one new Pdx$^-$ mutant, VS500 (*pdx-6*), two Arg$^-$ mutants, and two Cml$^-$ mutants among 2,000 Cys$^+$ colonies. This frequency of Cml$^-$ mutants (0.1%) was nearly twice that obtained by generalized mutagenesis (0.06% [1]). During its isolation, strain VS500 became stably lysogenized by SV1 and so could not be used subsequently as a donor. This was a relatively rare phenomenon for transductants that were routinely purified on citrate-supplemented agar. Similar hydroxylamine mutagenesis of VS500 (*pdx-6*) in turn and VS499 (*pdx-5*) gave two and one Cml$^-$ mutants, respectively. Linkage between these new *cml* mutations and *pdx-6* or *pdx-5* (and *cys-28*) remains to be confirmed, and more detailed analyses of their biochemical defects are in progress.

DISCUSSION

These cotransduction data show that the *cml* mutations analyzed so far are all located between *cys-28* and *pdx* loci. However, the relative order of mutation sites (Fig. 6) shows little correlation with sites of lesion within the chloramphenicol biosynthetic pathway (see Fig. 1). Thus, while *cml-6*, *-9*, and *-11* are located on one side of *cml-12* and give similar pleiotropic phenotypes (absence of any chloramphenicol precursors), another phenotypically similar mutation, *cml-7*, lies at the opposite end of the *cml* gene cluster. Possibly the former lie in a regulatory gene while the latter could be an early transcriptional stop mutation within a *cml* operon, or vice versa. On the other hand, the biochemically similar mutations *cml-1* and *cml-12* (both affecting arylamine synthetase) may be adjacent; also *cml-5* and *-8* (affecting hydroxylation of PAPA) could be adjacent, although *cml-4*, which apparently affects the same reaction (1), is separated from these loci at least by the site of *cml-12*.

In addition to their bearing on the relative order of some of the *cml* mutation sites, the present data also give information regarding the fine structure of *arg* and *pdx* loci. Thus, the *arg-4* mutation giving an Arg$^-$ phenotype is closely linked to *arg-5*, *-6*, *-12*, and *-16*, all of which give Orn$^-$ (or citrulline-negative) phenotypes; of the latter, *arg-16* may be more distant from the *cys-28* site than are the other *arg* mutations. We have also isolated *arg* mutations that do not appear to be contransducible with *cys-28*. Among the *pdx* mutations, only *pdx-5* failed to show cotransduction with *cys-28*; however, both *pdx-5* and *pdx-4* were cotransducible with *rib-2* (6), whereas *pdx-1*, *-2*, *-3*, and *-6* were not detectably cotransducible with *rib-2*. Therefore, *pdx-5* probably lies between *rib-2* and *pdx-4* at the end of the *pdx* locus furthest from the *cml* genes. The frequency of Cml$^+$ transductants in the recipro-

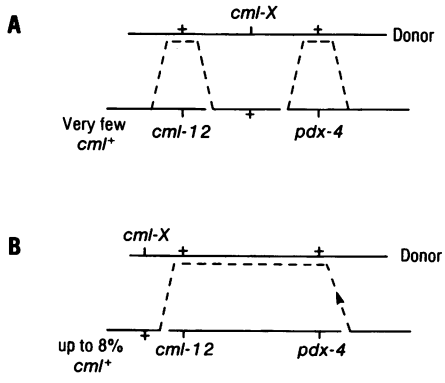

FIG. 4. Alternative arrangements of markers in a transduction between a *cml* donor and recipient strain VS258 (*cml-12 pdx-4*) (see Table 3, line 2).

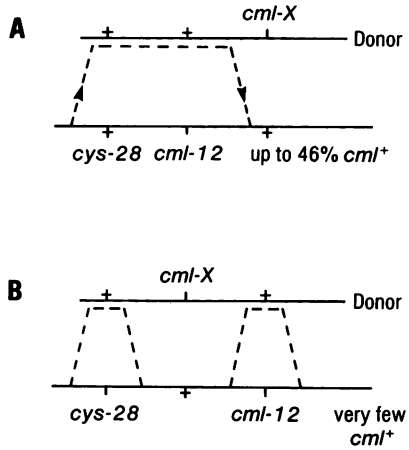

FIG. 5. Alternative arrangements of markers in a transduction between a *cml* donor and recipient strain VS263 (*cys-28 cml-12*) (see Table 3, line 3).

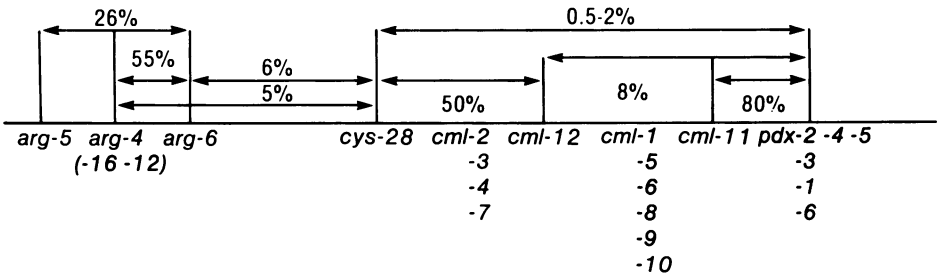

FIG. 6. Relative locations of *cml* markers between the cotransducible *cys-28* and *pdx* loci; percentages are cotransduction frequencies.

cal crosses VS253 × SV1 (VS499) (84%) and VS499 × SV1 (VS253) (100%) were consistent with the relative site order *cml-11–pdx-3–pdx-5*. The relative order of the remaining *pdx* mutations with respect to the *cml* genes may be discerned as more *cml*$^+$ *pdx* strains are constructed and used in three-point crosses with a *cml* mutation as the unselected marker. Unfortunately, we cannot directly select Cml$^+$ transductants from crosses involving donor and recipient Cml$^-$ mutants. Therefore, we cannot use *cys-28* or *pdx* mutations as unselected markers. By further strain construction to facilitate three-point crosses and by localized mutagenesis to induce new *cml* mutations and flanking markers, we aim to greatly improve this fine-structure analysis of *cml* genes in *S. venezuelae*. Such studies, possibly in conjunction with molecular cloning, should further elucidate the genetic organization and control of this secondary biosynthetic pathway.

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