FREQUENT LOSS AND RESTORATION OF ANTIBIOTIC PRODUCTION BY *STREPTOMYCES LASALIENSIS*

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Antibiotic nonproducing variants of *Streptomyces lasaliensis* NRRL 3382R, which makes the polyether antibiotic lasalocid A (Las) and the quinoxaline antibiotic echinomycin (Ech), arose at a frequency of 3~11% after treatment with three different mutagens or regeneration of protoplasts compared with a spontaneous frequency of <0.1%. Cosynthesis of lasalocid A was not observed upon testing a large number of Las$^-$ mutants in different pair-wise combinations, nor did these mutants accumulate probable intermediates of lasalocid A biosynthesis. These results suggest that loss of the *las* genes or their expression is induced at a high frequency by mutagenic treatments. In fusions of protoplasts of a strain with the *las*$^+$ *ech*$^+$ *spo*$^+$ *nic*-1 *rif*-3 markers with strains bearing the Las$^-$ Las^S Ech$^-$ Bld$^-$ (or *spo*$^+$) *str*-1 markers, Las$^+$ Ech$^+$ Spo$^+$ Str^R progeny were produced at a 61~89% frequency compared with a 1~9% frequency of Str^R antibiotic producing progeny with the *nic*-1 or *rif*-3 genotypes. The more frequent restoration of antibiotic production than prototrophy or rifampicin sensitivity indicates that these antibiotic characters did not behave as normal chromosomal markers. Therefore the genetic instability might be due to the involvement of a plasmid in antibiotic production. The apparent lack of infectious transfer of the Las$^+$ character to Las$^-$ parents in conjugal matings between the few strains tested and no correlation between the presence of a large plasmid, pKSL, and lasalocid A production in several strains of *S. lasaliensis* do not favor the latter hypothesis, but they do not conclusively disprove it. Consequently, we suggest that a plasmid or another mobile genetic element is controlling antibiotic production in *S. lasaliensis*.

Polyether antibiotics, produced by several species of *Streptomyces* and some other actinomycetes[1], are used as bovine growth promotants and coccidiostats[2]. The structural homology among polyether antibiotics[3] implies a functional similarity among the pathway enzymes in the different bacterial species which produce them[4]. It has been suggested that this relationship indicates a close similarity of the antibiotic production genes[3], as could also be true for the biosynthetically-related macrolide antibiotics[5]. Polyether antibiotics moreover are often produced in large amounts; a wild-type strain is known to have fermentation titers as high as 60 g/liter[6].

We have initiated a study of the genetics of polyether biosynthesis to uncover information about its enzymology and regulation, and report here our observations about the genetic instability of antibiotic production in *Streptomyces lasaliensis* which produces a polyether (lasalocid A (Las[†††])) and a quinoxaline (echinomycin (Ech)) antibiotic[1]. Las$^-$ strains of *S. lasaliensis* NRRL 3382R were generated at high frequency by mutagenesis and protoplast regeneration but *las* mutations did not

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[†††] Abbreviations used in the text: Ech; echinomycin, Las; lasalocid A, Str; streptomycin, Spc; spectinomycin, Rif; rifampicin, NTG; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 4-NQO; *N*-nitroquinoline-*N*-oxide, ^{S}; sensitive, ^{R}; resistant.

appear to block specific steps in the biosynthetic pathway. In protoplast fusions of some of these strains, antibiotic production was restored at high frequency but the Las$^+$ character was apparently not infectiously transferred to Las$^-$ strains in conjugal matings of other strains. Furthermore, the presence of a large plasmid pKSL, detected in the 3382R strain[7], was not fully correlated with lasalocid A production. These results point to the possibility that some other plasmid or mobile genetic element is controlling antibiotic production and responsible for the unusual behavior of *las* gene markers in *S. lasaliensis*.

Experimental

Bacterial Strains and Growth Conditions

The *S. lasaliensis* strains used in this study are listed in Table 1 and were grown on tomato paste agar (TPA) (tomato paste 20 g, Soyafluff 200W (Central Soya, Fort Wayne, IN) 10 g, Difco Bacto-peptone 1 g, glucose 10 g, sucrose 20 g, $CaCO_3$ 2 g, KH_2PO_4 1 g, agar 20 g and distilled water 1 liter), or maltose - malt extract - yeast extract (MYM) agar (maltose 4 g, Difco malt extract 10 g, Difco yeast extract 4 g, agar 20 g and distilled water 1 liter). TPA agar gave better sporulation than MYM agar, whereas antibiotic production was better on MYM agar than on TPA agar. The *Streptomyces griseus* 2682 and *bld*10 strains were obtained from JERRY ENSIGN, Department of Bacteriology, University of Wisconsin, Madison. All streptomycetes were grown at 30°C. *Bacillus subtilis* ATCC

Table 1. Strains of *Streptomyces lasaliensis* used in this study.

Strain	Genotype or phenotype	Source (mutagen)
3382R	Wild-type (*las*$^+$ *ech*$^+$ *spo*$^+$)	NRRL
JD 1	*las*$^+$ *ech*-4 *leu*-1 *spo*$^+$	HK 1 (NTG)
JD 2	*las*$^+$ *ech*-4 *pyr*-1 *spo*$^{+/-}$	HK 1 (NTG)
JD 3	*las*-173 *ech*-4 *ade*-1 *spo*$^{+/-}$	HK 1 (NGT)
JD 6	*las*-175 *ech*-4 *ser*-1 *spo*$^+$	HK 1 (UV)
JD 7	*las*-176 *ech*-4 *pyr*-2 *spo*$^+$	HK 1 (NTG)
HK 1	*las*$^+$ *ech*-4 *spo*$^+$	3382R (NTG)
HK 2	*las*$^+$ *ech*-4 *str*-1 *spo*$^+$	HK 1 (spontaneous)
HK 3	*las*$^+$ *ech*-4 *rif*-1 *spo*$^+$	HK 1 (spontaneous)
HK 4	*las*$^+$ *ech*-5 *spo*$^+$	3382R (UV)
HK 5	*las*$^+$ *ech*-5 *spc*-1 *spo*$^{+/-}$	HK 4 (spontaneous)
HK 19	*las*-57 Las^S *ech*-4 *str*-1 *spo*$^+$	HK 2 (UV)
HK 30	*las*-55 *ech*-4 *str*-1 *spo*$^{+/-}$	HK 2 (UV)
HK 46	*las*-81 *ech*-4 *str*-1 *spo*$^+$	HK 2 (NTG)
HK 60	Las$^-$ *ech*-4 Bld$^-$ *str*-1	HK 2 (NTG)
HK 108	Las$^-$ Las^S *ech*-4 Bld$^-$ *str*-1	HK 2 (protoplast regeneration)
HK 122	Las$^-$ Las^S *ech*-4 Bld$^-$	HK 2 (protoplast regeneration)
HK 132	*las*$^+$ *ech*$^+$ *nic*-1 *spo*$^+$	3382R (UV)
HK 133	*las*$^+$ *ech*$^+$ *nic*-1 *rif*-3 *spo*$^+$	HK 132 (spontaneous)
HK 134	Las$^-$ Las^S *ech*-6 *leu*-2 Bld$^-$ *spc*-2	3382R (UV→JD 8; spontaneous)
HK 136	*las*$^+$ *ech*-4 *ura*-1 *str*-1 *spo*$^+$	HK 2 (NTG)
HK 137	*las*-104 *ech*-4 *pro*-1 *str*-1 *spo*$^+$	HK 2 (NTG)
HK 138	*las*$^+$ *ech*-4 *lys*-1 *str*-1 *spo*$^+$	HK 2 (NTG)
HK 1062	*las*-177 *ech*$^+$ *spo*$^+$	3382R (UV)
HK 1063	*las*-178 *ech*$^+$ *spo*$^+$	3382R (UV)
HK 1064	*las*-179 *ech*$^+$ *spo*$^+$	3382R (UV)
SO 1	*las*$^+$ *ech*-1 *spo*$^+$	3382R (NTG)
SO 2	*las*$^+$ *ech*-2 *spo*$^+$	3382R (NTG)

spo$^{+/-}$: Poor sporulation.
Las^S: Lasalocid A-sensitive as defined in the Experimental.
Bld$^-$: Bald.

27859 was grown at 37°C on Difco Antibiotic Medium No. 1.

Mutagenesis Protocols

Suspensions containing 10^7 to 10^9 spores/ml of *S. lasaliensis* were mutagenized with UV light or NTG using the procedures described by HOPWOOD *et al.*[8]. Mutagenesis with 4-NQO was carried out as described for NTG[8]. Single colonies from aliquots in which 98~99.8% of the spores had been killed by the mutagenic agent were transferred to TPA master plates and MYM agar plug plates[9], and grown for 5 to 9 days before assaying antibiotic production or scoring other phenotypes. All mutants were transferred at least once before these assays to allow phenotypic segregation. Spontaneous drug-resistant mutants were selected by growing strains on MYM agar containing spectinomycin (100 μg/ml) or streptomycin (25 μg/ml); or by first growing strains in liquid MYM containing rifampicin (25 μg/ml) for 5 days, then plating mycelia obtained from this culture on MYM agar containing the same concentration of rifampicin.

Protoplast Regeneration

Strains were grown in baffled flasks in Difco tryptic soy broth containing 0.5% glycine to an A_{600} value of *ca.* 5, then protoplasts were prepared by the method of SHIRAHAMA *et al.*[10] using their P3 and PWP buffers. Protoplasts were regenerated on dried R2YE plates overlaid with soft R2YE agar according to the method of HOPWOOD *et al.*[8].

Assays of *S. lasaliensis* Mutants for Antibiotic Production, Resistance and Cosynthesis

Production of echinomycin and lasalocid A by strains grown on agar plugs was measured by bioassay with *B. subtilis* (Las^S Ech^S), with the echinomycin-sensitive *S. lasaliensis* 58 strain, or with the echinomycin-resistant strain of *Streptomyces venezuelae* NRRL 10712 that was sensitive to 1 μg/ml of lasalocid A. Strains showing no zone of growth inhibition were retested after single colony purification to confirm the antibiotic nonproduction phenotype. To identify Las^+ Ech^- and Las^- Ech^+ mutants, or to reconfirm the antibiotic nonproducing character of strains, bioautography was carried out by chromatography of ethyl acetate extracts, made from 5 ml cultures of strains grown in liquid MYM medium, on silica gel in ethyl acetate, or in hexane - 2-propanol - acetic acid (85 : 15 : 0.5), followed by bioassay of the air-dried plate with *B. subtilis*. Strains that maintained their antibiotic nonproduction phenotype after 5 to 10 serial transfers were considered to be stable mutants.

Cosynthesis of antibiotic activity was determined in one or more of the following ways. (i) Cross streaking on MYM agar plates and, after incubation, overlaying the plates with *B. subtilis* seeded medium. (ii) Growth of two different mutants in the same MYM agar plug followed by bioassay with *B. subtilis*. (iii) Mixed culture of two different mutants in 5 ml of liquid MYM medium followed by bioassay of the culture broth using filter discs or bioautography. (iv) Extraction of a 5-ml liquid MYM culture of one mutant with ethyl acetate, evaporation of the extract to dryness and dissolving the residue in 1.5 ml of dimethyl sulfoxide, followed by addition of a 100-μl aliquot of this solution to a 6-day old 5 ml liquid MYM culture of another mutant. The culture broth was then bioassayed after 4 more days of incubation.

Lasalocid A-resistance was determined by testing the ability of Las^- mutants to grow on MYM agar containing 50 μg/ml of lasalocid A. Strains that failed to grow were assigned the Las^S phenotype. The echinomycin-resistance of mutants was not determined.

Detection and Analysis of Compounds Accumulated by Las^- Mutants

Mutants were grown in 5 ml of liquid MYM medium for 7 days and the culture broth and mycelia were extracted with 1 ml of ethyl acetate by vortexing. A 30-μl aliquot of this extract was chromatographed on silica gel in ethyl acetate. The dried chromatogram was sprayed with a solution of vanillin (3 g), concentrated sulfuric acid (0.5 ml) and ethanol (100 ml), then heated to 110°C for a few minutes until the coloration of various spots was strongly developed.

Strain HK 30 was grown in 4 liters of liquid MYM medium and compounds I and II were isolated by extraction of the culture with ethyl acetate and chromatography of the concentrated extract on Sephadex LH-20 in methanol. Fractions from this column containing the desired compounds were combined and evaporated, and the resulting residue was chromatographed on silica gel plates

in ethyl acetate. Extraction of the silica gel containing the appropriate bands gave 6.6 mg of compound I and 7.4 mg of compound II. The MS data and ^{1}H and ^{13}C NMR spectral data for compounds I and II were obtained. The spectral data for compound I indicated that it was anthranilamide. Refluxing compound I or anthranilic acid in methanolic HCl gave methyl anthranilate. The spectral and optical rotation data for compound II were identical with the values reported for it by KELLER-SCHIERLEIN *et al.*[11].

Conjugal Matings of *S. lasaliensis* Strains

Auxotrophs of *S. lasaliensis* strains were isolated and classified by methods similar to those described by WEBER *et al.*[9], using the HOPWOOD minimal medium[8]. Equal numbers of spores from the two parental strains were mixed and grown on TPA plates for 7 days, then the harvested spores were grown nonselectively and analyzed for the parental phenotypes to determine if a growth imbalance was present. Selection for prototrophs was done by growth on minimal medium or for a particular phenotype by growth on supplemented minimal medium following standard methods[8,9]. Lasalocid A production by recombinants was assayed on MYM agar as described above.

Protoplast Fusions of *S. lasaliensis* Strains

Protoplasts of the two parental strains were mixed in the stated ratio (Table 5) and washed once with PWP buffer by centrifugation at 3,000 rpm for 10 minutes. The pelleted protoplasts were gently resuspended in the small amount of liquid remaining in the centrifuge tube, then 400 μl of a 50% solution of polyethylene glycol 1000 (Koch-Light Ltd.) in PWP buffer was added and the protoplasts were mixed gently by pipetting up-and-down several times. After 2 minutes at room temperature, the mixture was serially-diluted in PWP buffer, applied to air-dried R2YE plates and overlaid with soft R2YE agar. After 2 days of incubation, Str^R or Spc^R colonies were selected by overlaying the regeneration plates with soft Difco nutrient agar containing sufficient streptomycin or spectinomycin to give a final concentration of 25 or 100 μg/ml, respectively. After 3 days further growth, the resulting colonies were transferred to TPA master plates and *ca.* 100 of them screened as follows: Antibiotic production was measured by the agar plug and bioautography methods described above; prototrophy by growth on the HOPWOOD minimal medium; sporulation by growth on TPA agar; and rifampicin resistance by growth on MYM agar containing 25 μg/ml of rifampicin. The stability of the antibiotic production phenotype was determined by serial transfer on TPA plates followed by bioassay of MYM agar plugs. As controls, protoplasts of each parental strain were carried through the same procedure to show that the reversion frequency of parental markers was $<1\%$.

Responsiveness of *S. lasaliensis* Strains to A Factor

This was determined by co-streaking the *S. griseus* 2682 (A factor$^+$) and *bld*10 (A factor-sensitive) strains adjacent to *S. lasaliensis* strains on TPA plates and incubating for 7 days.

Results

Isolation and Cosynthetic Characterization of Antibiotic Nonproducing Mutants

We were primarily interested in studying Las$^-$ strains and assumed that Las$^-$ Ech$^-$ double mutants would be isolated at a low frequency after a single mutagenesis, unless a mutation having a pleiotropic effect on antibiotic production were easily induced in *S. lasaliensis*. Therefore, Las$^+$ Ech$^-$ strains of *S. lasaliensis* were isolated first in one of two ways. Bioassay of mutagenized spores from the NRRL 3382R strain with an echinomycin-sensitive strain of *S. lasaliensis* and then with *B. subtilis* (Las^S) identified the resulting Las$^+$ Ech$^-$ mutants. Alternatively but less effectively, mutagenized strains that first were shown to have weak antibiotic activity against *B. subtilis* were screened with the same two bioassays to identify Las$^+$ Ech$^-$ mutants. The Las$^+$ Ech$^-$ character of the mutants was confirmed by TLC and bioautography of ethyl acetate extracts of liquid cultures. Las$^-$ Ech$^+$ mutants were isolated by bioassay with an echinomycin-resistant, lasalocid A-sensitive strain of *S. venezuelae* and

Table 2. Frequency of isolation of Las$^-$ Spo$^+$ mutants of *Streptomyces lasaliensis*.

	SO 1		SO 2		3382R[a]	
	NTG	UV	NTG	UV	UV	UV
Las$^-$ Spo$^+$ strains[b]; first assay (%)	3.3 (32[c])	4.4 (43)	7.9 (61)	9 (87)	2.5 (24)	5.5 (53)
Las$^-$ Spo$^+$ after restreaking once	0.6	2.1	1.8	3.7	1.4	1.4
Las$^-$ Spo$^+$ after restreaking twice	0.3	1	0.7	2.3	0.7	0.7

[a] The mutants of this strain were Las$^-$ Ech$^+$.
[b] Growth of the mutants isolated from the SO 1 and SO 2 parentals was not inhibited by 10 μg/ml of lasalocid A; the mutants isolated from the 3382R parent were not tested for lasalocid A sensitivity.
[c] Number of mutants initially isolated.

then with *B. subtilis*.

In the first search for Las$^-$ mutants, UV or NTG-mutagenesis of two Las$^+$ Ech$^-$ strains and the wild-type strain resulted in the isolation of Las$^-$ Ech$^-$ mutants of strains SO 1 and SO 2, and Las$^-$ Ech$^+$ mutants of NRRL 3382R, at frequencies of 2.5 to 9.0% (Table 2) (Las$^-$ Ech$^-$ (or Ech$^+$) mutants formed spontaneously from either of these three parental strains were isolated at a frequency of $<0.1\%$). The majority of the sporulating (Spo$^+$), Las$^-$ Ech$^-$ (or Ech$^+$) mutants initially isolated produced bald (Bld$^-$; *i.e.*, no aerial mycelia) segregants or Las$^+$ revertants at high frequencies as reflected in Table 2 by the decreasing percentages of Las$^-$ Spo$^+$ mutants recovered upon serial transfer. By picking colonies with a wild-type appearance from the serial transfers, we isolated 54 stable Las$^-$ Ech$^-$ (or Ech$^+$) Spo$^+$ strains at an overall frequency of 1.0%. None of these mutants exhibited lasalocid A cosynthesis when tested in pair-wise combinations by four different methods (Experimental section).

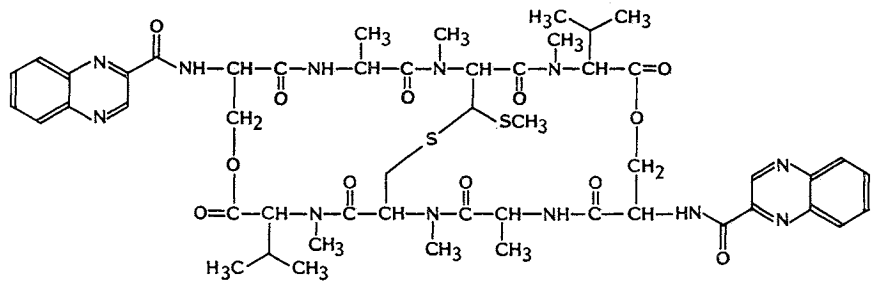
A second search for Las$^-$ mutants was carried out using a less stringent protocol. A Las$^+$ Ech$^-$ strain with wild-type morphology, HK 1, was marked with streptomycin-resistance (HK 2) by spontaneous mutation at a frequency of *ca.* 2×10^{-6}. The Las$^-$ Ech$^-$ mutants of HK 2, isolated at frequencies of 7.5 to 11.5% following treatment of spores with UV, NTG or 4-NQO, or protoplast regeneration, were identified as before but without exclusion of the ones with abnormal colony morphology (24% of the mutants had abnormal colony morphology and 7% were Las^s). Some of the Las$^-$ mutants were also Bld$^-$; these probably contained a mutation that indirectly resulted in the loss of lasalocid production and development of aerial mycelia. Therefore, we have used phenotypic designations for these mutations in the appropriate strains (Table 1).

One hundred and nine Las$^-$ Ech$^-$ mutants of HK 2, representative of the phenotypes determined by the following tests, were chosen for further study: Colony morphology on TPA plates, TLC analysis of ethyl acetate extracts of liquid cultures, and sensitivity to lasalocid A. Upon testing for antibiotic cosynthesis in all of the 5,886 possible pair-wise combinations by the agar plug method (Experimental section), no positive combination was found for any of these mutants.

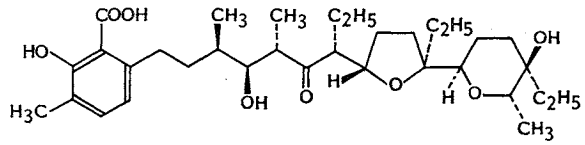
Characterization of the Metabolites Accumulated by Las$^-$ Strains

Solvent extracts of liquid cultures of all of the 370 Las$^-$ Ech$^-$ mutants of HK 2 were examined by a chemical screen[12] to determine if any strains had accumulated possible intermediates of lasalocid A biosynthesis. The extracts of many of these strains had a common TLC profile (silica gel developed with ethyl acetate) consisting mainly of three colored spots (yellow (compound I, Rf 0.56), red (compound II, Rf 0.40) and blue (compound III, Rf 0.24)) after treatment with a chromogenic spray reagent.

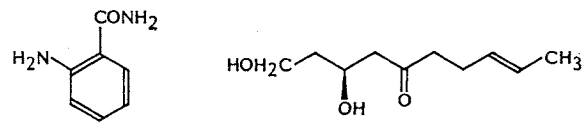
Fig. 1. Compounds produced by the wild-type strain or by Las⁻ mutants of *Streptomyces lasaliensis*.



Echinomycin



Lasalocid A



I II

The same compounds were detected in smaller amounts in extracts of some of the Las⁺ strains, however.

The metabolites accumulated by a representative Las⁻ Ech⁻ strain (HK 30) were isolated and two of them characterized. Compound I was identified as anthranilamide by spectral and chemical comparisons. Compound II was found to be (3*S*,8*E*)-1,3-dihydroxy-8-decen-5-on by comparison with the physical and chemical data reported for this substance[11], which had been previously isolated from *Streptomyces fimbriatus*. Based on what is known about lasalocid A biosynthesis[4,13~16], neither compound I nor II resemble likely intermediates of this pathway. We also doubt that they are distantly related shunt metabolites because their structures (Fig. 1) lack the characteristic *C*-methyl or *C*-ethyl groups present in lasalocid A and other polyether antibiotics[3].

The above conclusion and the lack of antibiotic cosynthesis by a large number of Las⁻ Ech⁻ strains indicate that antibiotic nonproduction apparently did not result from the blockade of specific steps in the lasalocid A pathway, if in fact such blocks would have caused the accumulation of diffusible and biotransformable intermediates; or that it was caused by a blockade of the same (early) step in this pathway which did not then result in the accumulation of pathway metabolites. It is more likely that the mutagenic treatments stimulated a genetic instability of *S. lasaliensis* which resulted in the inactivation of the same (early) *las* structural gene or another gene regulating expression of the *las* structural genes, or in the loss of all of the *las* genes at a high frequency. These possibilities were

tested by the following experiments.

Genetic Recombination of *las* Genes by Conjugal Mating

We mutagenized *S. lasaliensis* with UV or NTG, isolated auxotrophs at a frequency of 3.4 to 6.6%, and determined the Las$^+$ or Las$^-$ phenotype and nutritional requirement of nine auxotrophic strains with reversion frequencies of $<1.1\times10^{-5}$. Using several of these strains in conjugal matings, we selected for prototrophic recombinants in two-factor crosses and obtained recombination frequencies of 1.0×10^{-1} to 3.8×10^{-3} (data not shown). These frequencies are higher than typically found for conjugal matings of streptomycete strains[17]. The spores recovered from conjugal matings of three suitably marked strains were grown on minimal media supplemented with the appropriate amino acid to counterselect the Las$^+$ (crosses 1 and 3) or Las$^-$ (crosses 2 and 4) parents and the progeny scored for the four possible genotypes. The results (Table 3) do not reveal any obvious linkage between the *las* characters and the nonselected auxotrophic markers because an approximately equal number of Las$^+$ recombinants were *ser*$^+$ or *ser* (cross 1) and *ade*$^+$ or *ade* (cross 3), and Las$^-$ recombinants were *leu*$^+$ or *leu* (crosses 2 and 4). When these data were analyzed by the method of HOPWOOD[18,19], they did not strongly support infectious transfer of the *las* genes. Only 23 to 48% of the Las$^-$ parentals (crosses 1 and 3) were "infected" with the Las$^+$ character (as defined for SCP1

Table 3. Results of conjugal matings of *Streptomyces lasaliensis* strains.

Cross	Strains	Genotypes scored			Number of each genotype isolated
		ser	*leu*	*las*	
1[a]	JD 1×JD 6	−	+	+	16
		+	+	+	15
		−	+	−	54 (parental)
		+	+	−	15
	las$^+$ recombinants[d]=67%, *ser leu*$^+$ *las*$^+$ recombinants[d]=35%				
2[b]	JD 1×JD 6	+	−	+	4 (parental)
		+	+	+	71
		+	−	−	13
		+	+	−	14
	las recombinants[d]=27%, *ser*$^+$ *leu las* recombinants[d]=13%				
		ade	*leu*	*las*	
3[a]	JD 1×JD 3	−	+	+	27
		+	+	+	38
		−	+	−	29 (parental)
		+	+	−	6
	las$^+$ recombinants[d]=92%, *ade leu*$^+$ *las*$^+$ recombinants[d]=38%				
4[c]	JD 1×JD 3	+	−	+	6 (parental)
		+	+	+	19
		+	−	−	31
		+	+	−	46
	las recombinants[d]=80%, *ade*$^+$ *leu las* recombinants[d]=32%				

[a] *leu*$^+$ colonies selected by growing spores recovered from the mated strains on minimal media (MM) supplemented with serine; data are the average of three crosses.

[b] *ser*$^+$ colonies selected on MM+leucine as in [a].

[c] *ade*$^+$ colonies selected on MM+leucine as in [a].

[d] Total number of colonies of this genotype isolated divided by [(the total number of colonies isolated)−(the number having the parental genotype)]×100.

JD 1 (*ser*$^+$ *leu*$^-$ *las*$^+$); JD 6 (*ser leu*$^+$ *las*); JD 3 (*ade leu*$^+$ *las*).

and the methylenomycin A genes, true infectious transfer should have occurred at a much higher frequency, close to 100%[18]), and 76 to 84% of the Las$^+$ parentals (crosses 2 and 4) lost their Las$^+$ characteristic (which is the opposite of infection[18]).

If the *las* genes were linked to a plasmid sex factor such that Las$^-$ strains, having lost the plasmid, would have exhibited a markedly diminished ability to undergo recombination in conjugal matings, as in the case of SCP1[18]), the recombination frequencies in Las$^-$ × Las$^-$ crosses should have been much lower than in Las$^+$ × Las$^-$ crosses. Using different combinations of *las*$^+$ or *las*$^-$ alleles in two-factor crosses between several auxotrophic strains, we observed that the Las$^-$ × Las$^-$ crosses were generally less fertile than Las$^+$ × Las$^+$ and Las$^+$ × Las$^-$ crosses (Table 4). These differences, however, are much less than was seen in crosses between *Streptomyces coelicolor* fertility types in which the recombination frequency varied as much as 10^5 depending on the presence or absence of SCP1[18]). In a cross between two Las$^-$ auxotrophs, JD 6 and JD 7, 2 of 94 prototrophic recombinants were Las$^+$. Restoration of lasalocid A production by homologous recombination is only possible in this experiment if the *las* mutations in the parental strains are not large deletions. Mating of course might have induced reversion of either of the *las* markers, but the reversion frequency of all the Las$^-$ strains studied was shown to be $<0.1\%$.

When the above results are compared with the *S. coelicolor*/SCP1 system[18]), the lack of infectious transfer of the Las$^+$ characteristic at the high frequencies reported for SCP1$^+$ × SCP1$^-$ crosses[18]) and the only moderate decrease in the fertility of Las$^-$ × Las$^-$ crosses do not point to the presence of the *las* genes on a self-transmissible plasmid, though a plasmid-linked mutation rather than plasmid loss could still be invoked. To test if the *las* gene markers were located on a non-transmissible plasmid, the following experiments were done.

Genetic Recombination of *las* Genes by Protoplast Fusion

Using a different set of genetically marked strains in protoplast fusion experiments, the recom-

Table 4. Results of conjugal matings of *Streptomyces lasaliensis* Las$^{+/-}$ strains.

Cross	Strains	Frequency of prototrophic recombinants[a]
Las$^+$ × Las$^+$:		
5	JD 1 × JD 2	1.0×10^{-1}
6	HK 138 × JD 1	3.8×10^{-3}
7	HK 138 × HK 136	1.9×10^{-3}
Las$^+$ × Las$^-$:		
8	JD 1 × JD 6	3.6×10^{-2}
9	JD 2 × JD 6	1.6×10^{-2}
10	JD 1 × JD 3	1.2×10^{-2}
Las$^-$ × Las$^-$:		
11	JD 6 × HK 137	2.1×10^{-3}
12	JD 6 × JD 7	4.1×10^{-4}
13	JD 3 × HK 137	4.2×10^{-5}

[a] Number of colonies appearing on minimal medium divided by the number of colonies appearing on complete medium per unit volume of a suspension of the recovered spores.

Table 5. Results of protoplast fusions of *Streptomyces lasaliensis* strains.

Cross	Strains	Ratio of protoplasts[a]	Frequency or recombinants (%)
14	HK 132 × HK 138	1	0.6[b]
15	JD 1 × HK 138	0.8	3.3[b]
16a	HK 133 × HK 108	100	89.5[c]
16b	HK 133 × HK 108	20	77.9[c]
16c	HK 133 × HK 108	5	76.8[c]
16d	HK 133 × HK 108	1	61.5[c]
17	3382R × HK 108	20	52.4[c]
18	HK 133 × HK 19	20	80.4[c]
19	HK 3 × HK 108	5	29.3[c]
20	HK 133 × HK 134	20	9.9[d]

[a] Number of protoplasts/ml of the first strain divided by the number for the second strain.
[b] Prototrophic recombinants (the value calculated as described in Table 4 × 100).
[c] Antibiotic producers among the Str^R colonies.
[d] Antibiotic producers among the Spc^R colonies.

bination frequency of auxotrophic markers was found to be 6 to 33×10^{-3} (Table 5, crosses 14 and 15), which is comparable with the results found in the conjugal matings (4 to 100×10^{-3}) and protoplast fusions of other *Streptomyces*[20,21]. Fusions were carried out between different ratios of protoplasts of different Las$^+$ Ech$^+$ or Las$^+$ Ech$^-$ and Las$^-$ Las^S Ech$^-$ strains by using the *str*-1 or *spc*-2 resistance of the Las$^-$ Las^S Ech$^-$ parent to counterselect the Las$^+$ parent (Table 5). The resulting progeny were scored for antibiotic production, drug resistance, nutritional requirement and sporulation ability. In the four variations of cross 16, 61 to 89% of the streptomycin-resistant colonies were antibiotic producers; among these, 90% produced both antibiotics and 10% produced only lasalocid A. Of the antibiotic producers from cross 16b, 90% were Spo$^+$, 92% were prototrophic and 99% were Rif^S. Thus most of the antibiotic producing recombinants were Las$^+$ Ech$^+$ Spo$^+$ *str*-1, indicating that the Las$^-$ Las^S Ech$^-$ parent had gained several properties (Las$^+$, Las^R, Ech$^+$, Spo$^+$) from the Las$^+$ Ech$^+$ parent at high frequency. Since the majority (>91%) of the progeny analyzed from cross 16b did not have the nicotinic acid requirement or rifampicin-resistance of the counterselected parent, the streptomycin-resistant progeny were not merely Str^R mutants of HK 133; therefore, *str*-1 antibiotic producing, *nic*-1 or *rif*-3 recombinants represented less than 9% of these progeny. When cross 16b was analyzed nonselectively, instead of for Str^R progeny, 96% of the colonies had the phenotype of the HK 133 parental strain, which establishes the stability of its genetic characteristics and reflects the effect of the 20:1 ratio of parenteral strains in this cross. The result of cross 17 was similar to cross 16b, and the result of cross 18 shows that the transfer of the antibiotic producing property was not linked with the sporulation ability of the Las$^-$ Las^S parent. The data from crosses 19 and 20 indicate that the apparently high frequency transfer of the Las$^+$ property was strain dependent. Since the antibiotic producing ability of approximately 20 colonies chosen from crosses 19 or 20 was unstable (62~70% lost production upon restreaking), in contrast to those taken from cross 16b (only 5% lost production), the majority of the antibiotic producers recovered from crosses 19 and 20 may have been heterokaryons[19] rather than recombinants. This could be the reason for the apparently low frequency transfer of the Las$^+$ character in these two crosses.

The effect of the following two things on the results of the protoplast fusions was not directly addressed. Strains HK 19, HK 108, and HK 134 used in crosses 16~20 are Las$^-$ Las^S. If the Las$^+$ colonies produced in these fusions had inhibited the growth of Las^S progeny, this could have resulted in artificially high recoveries of Las$^+$ Las^R recombinants. However, stable Las$^-$ Las^S strains were recovered in crosses 16~18, suggesting that an apparent unidirectional transfer of the *las* genes took place in these crosses. Secondly, the Las$^-$ and Bld$^-$ mutations in strains HK 108 and HK 134 were introduced simultaneously by protoplast regeneration or UV mutagenesis, respectively. If this comutation indicates that these two characteristics resulted from the indirect effect of a mutation lying outside of the *las* or *bld* gene loci, the frequent restoration of lasalocid A production and sporulation in crosses 16 and 17 may not have been due to the transfer of *las* and *bld* genes. This disclaimer is countered by the results of cross 18, however, since the recipient strain HK 19 was Spo$^+$.

Correlation of Antibiotic Production with the Presence of a Plasmid

The results of the protoplast fusions described above might be explained by the unidirectional, high frequency transfer of plasmid borne *las* genes unaccompanied by significant recombination between presumed chromosomal markers. For this reason, we investigated the relationship between a large (*ca.* 520 kilobase pairs) plasmid, pKSL, recently discovered in the wild-type *S. lasaliensis*[7],

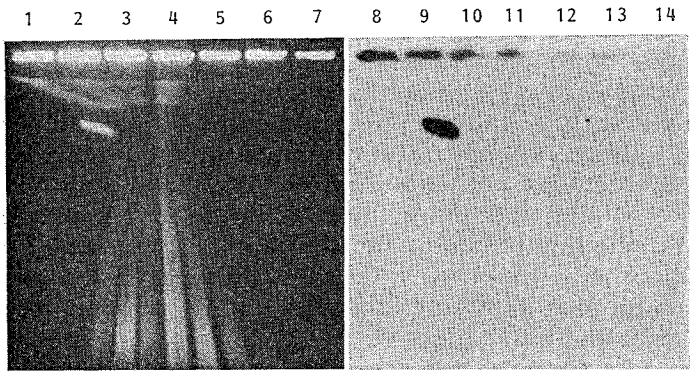
Table 6. Relationship between antibiotic production and presence of plasmid pKSL among *Streptomyces lasaliensis* strains.

Strain	Antibiotic production		Presence of pKSL[a]
	Lasalocid A	Echinomycin	
3382R	+	+	+
HK 133	+	+	+
HK 1	+	−	−[b]
HK 2	+	−	−[b]
HK 3	+	−	−[b]
HK 4	+	−	−[b]
HK 19	−	−	−
HK 46	−	−	−
HK 60	−	−	−
HK 108	−	−	−
JD 8[c]	−	−	+
HK 1062	−	+	+
HK 1063	−	+	+
HK 1064	−	+	+
Fusant from 3382R × HK 108[d]	+	+	+
Fusant from HK 133 × HK 108[d]	+	+	+
Fusant from HK 133 × HK 60[e]	+	+	+
Fusant from HK 133 × HK 19[e]	+	+	+
Fusant from HK 133 × HK 46[e]	+	+	+
Fusant from HK 3 × HK 108[d]	+	−	−[b]
Fusant from HK 1062 × HK 108[d]	−	+	+

[a] Determined as described in ref 7.
[b] Confirmed by Southern hybridization of total DNA with [^{32}P]pKSL (Fig. 2).
[c] The Spc^S parent of HK 134.
[d] Two colonies were tested.
[e] One colony was tested.

Fig. 2. OFAGE (lanes 1~7) and Southern hybridization (lanes 8~14) of total DNA isolated from *Streptomyces lasaliensis* strains.

Lanes 1 and 8, NRRL 3382R; 2 and 9, HK 1; 3 and 10, HK 2; 4 and 11, HK 4; 5 and 12, HK 3; 6 and 13, a progeny from cross 19 of Table 5; 7 and 14, the second progeny from the same cross.



Total DNA was isolated and OFAGE was done with 1.5% agarose in 0.5X TBE at 300 V with a switching interval and operation time of 30 seconds and 24 hours, respectively, as described in ref 7.

and antibiotic production. pKSL is absent in strain HK 108 and reappears in the Las$^+$ Ech$^+$ Spo$^+$ progeny obtained from cross 16 of Table 5[7]. Several other *S. lasaliensis* strains were screened for the presence of pKSL, and a correlation between its presence and lasalocid A production was found in several cases (Table 6). Yet pKSL was detected in some Las$^-$ strains (JD 8, the Spc^S parent of HK 134; HK 1062~1064), and it could not be detected in several Las$^+$ strains (HK 1~4) even by Southern hybridization of total DNA from these with [^{32}P]pKSL (Fig. 2). Similarly, pKSL was present in a nonsporulating strain (JD 8), and absent from some sporulating strains (HK 1~4, HK 19, and HK 46). There was a better correlation between the presence of pKSL and echinomycin production, with strain JD 8 being the only exception (the Ech$^-$ phenotype of JD 8 could be due to a genuine point mutation). pKSL was transferred to the antibiotic nonproducing strains in six protoplast fusion experiments. The fusion of HK 3 and HK 108 showed that pKSL was not detected in the progeny of a cross between two pKSL$^-$ strains. Despite the fact that the progeny in which pKSL could be detected all produced an antibiotic and sporulated, the presence of pKSL clearly was not necessary for lasalocid A production; therefore the *las* genes do not appear to reside on this plasmid. No other plasmids were detected in *S. lasaliensis* strains[7].

Possible Involvement of a Low Molecular Weight Regulator in Antibiotic Production

Streptomyces commonly produce A factor, a low molecular weight compound that regulates the formation of aerial mycelia, spores, and antibiotics or pigments in some strains[22]. Incubation of *S. griseus* 2682, an A factor secreting strain, close to *S. lasaliensis* HK 108 did not cause the latter to sporulate, unlike the control strain, *S. griseus bld*10, which responded normally to A factor. The latter strain was also induced to sporulate when incubated close to *S. lasaliensis* HK 132 but not to HK 108. HK 132 did not induce sporulation or antibiotic production in HK 108 when these two strains were incubated at suitable distances on TPA plates. Thus a substance with A factor-like activity appears to be secreted by a morphologically normal, antibiotic-producing *S. lasaliensis* strain (HK 132); but a bald, antibiotic nonproducing strain (HK 108) neither produced A factor nor responded to it.

Discussion

The high frequency induction of mutations which block antibiotic production by *S. lasaliensis* is not unusual because this genus is noted for instability of antibiotic production, drug resistance, sporulation and some other properties[23]. Nonetheless, it is striking that protoplast regeneration produced Las$^-$ mutants at the same frequency as treatment with UV, NTG or 4-NQO. This kind of result has been taken as an indication for the involvement of a plasmid in antibiotic production because protoplast regeneration can cause a frequent loss of bacterial plasmids[24], including SCP1, the self-transmissible plasmid of *S. coelicolor* that carries the methylenomycin A production and resistance genes[24,25]. Protoplast regeneration can also cause DNA amplification or deletion in *Streptomyces*[23], either of which could have inactivated or resulted in the loss of one or more *las* genes. Therefore, alternative explanations of our results must be considered before the loss of a plasmid.

It is unlikely that a pleiotropic A factor$^-$ mutation induced at high frequency and causing the loss of aerial mycelia or sporulation, which are developmentally regulated properties usually connected with antibiotic production in *Streptomyces*[26], caused the facile loss of antibiotic production in most cases because Las$^-$ mutants with normal morphology were often isolated. This and the finding that a typical bald, Las$^-$ mutant was not responsive to the sporulation and antibiotic production-inducing effects of A factor[22] indicate that even though *S. lasaliensis* seems to secrete A factor-

like activity, loss of this property does not appear to be the reason for the frequent production of Las$^-$ mutants among the strains we tested.

Lasalocid A biosynthesis could involve only enzyme-bound or non-diffusible intermediates. In this case, strains with *las* mutations anywhere in the large region of DNA required for this multi-step pathway would exhibit a non-cosynthesizing, Las$^-$ phenotype in cofermentations. Because of insufficient information about the enzymology of polyether biosynthesis[4,27], we cannot discount this as an explanation for the lack of cosynthesis by Las$^-$ mutants. Furthermore we have not been able to demonstrate cosynthesis between strains with mutations that block the biosynthesis of two other polyether antibiotics made by *Streptomyces*: Monensin (M. J. DONOVAN *et al.*; unpublished results) and salinomycin (C. W. BORELL *et. al.*, and C. R. HUTCHINSON; unpublished results).

Amplification of a DNA segment, a general property of some other *Streptomyces*[28], could have been induced at high frequency and caused the loss of *las* gene expression directly, or indirectly by affecting the determinants of cellular differentiation. Such mutants would not exhibit antibiotic cosynthesis nor accumulate intermediates of the lasalocid A pathway. Efficient restoration of the normal genome by recombination in the protoplast fusion experiments could then have been the reason for the apparent high frequency recovery of antibiotic production and sporulation by strain HK 108, for example. This idea is not supported by our preliminary observations that gel electrophoresis of the *Bam*H I digested total DNA isolated from different Las$^+$ or Las$^-$ *S. lasaliensis* strains, including HK 2, HK 5, and HK 108, did not show distinct amplified DNA fragments, although discrete DNA fragments were seen in HK 122 (H. KINASHI and C. R. HUTCHINSON; unpublished data). We therefore doubt that the facile production of Las$^-$ mutants and restoration of lasalocid A production by protoplast fusion were related to DNA amplification, but this matter merits further study.

High frequency induction of large DNA deletions removing the majority of the *las* genes is eliminated as a characteristic of several of the Las$^-$ mutants by the results of our two-factor crosses. But if the *las* genes were clustered like several other sets of antibiotic genes in the *Streptomyces*[28], a frequently-induced smaller deletion or rearrangement of a DNA segment near or within this gene cluster (such as transposition of a mobile genetic element), if it interrupted expression of a set of cotranscribed *las* genes, could have had the same effect on the production of Las$^-$ mutants and the outcome of the protoplast fusion experiments as DNA amplification. This possibility of course can be tested when cloned *las* genes become available.

Since none of the above explanations are uniquely supported by the present data, the role of a plasmid that carries the *las* genes or controls their expression can now be considered more thoroughly.

The involvement of plasmid DNA in antibiotic biosynthesis has been claimed in several instances[17], but proven only for the case of methylenomycin A[7,25,29]. Acceptable evidence is the demonstration that antibiotic production genes are not linked to chromosomal genes, that the antibiotic production characteristic can be transferred from one to another strain at a frequency much different than the recombination frequency of chromosomal markers, or that a plasmid shown to bear the antibiotic production genes (*e.g.*, by the introduction of cosynthetically permissive mutations which are plasmid linked) can be isolated. Expression of the antibiotic production genes following introduction of this plasmid into a heterologous background, as for methylenomycin A[25], provides the ultimate proof.

With these criteria in mind, some of our results favor plasmid DNA involvement in the production of lasalocid A by *S. lasaliensis*. Antibiotic production and sporulation were restored at a much higher frequency than chromosomal recombination took place in protoplast fusions (crosses 16 and 18 of Table 5) because *nic*-1 *str*-1 or *rif*-3 *str*-1 antibiotic producers were recovered 9 to 99-fold less frequently than Las$^+$ Ech$^+$ Spo$^+$ *str*-1 progeny. It the *rif* and *str*-resistance genes lie close together in *S. lasaliensis*, as they do in *S. coelicolor*[17], this could explain why *rif*-3 *str*-1 recombinants were rarely encountered but not why *nic*-1 *rif*-1 recombinants were rare. CHATER and HOPWOOD have noted that chromosomal recombination in fusions often occurs at a high frequency such that apparently parental progeny are actually recombinants[17]. In this case, efficient recombinational repair of the *las* and *bld* mutations, instead of plasmid transfer, could have restored antibiotic production and sporulation in the Las$^+$ Ech$^+$ Spo$^+$ progeny from the crosses in Table 5. Thus the available data can only suggest that the

las genes were transferred to Las$^-$ strains at a frequency higher than the recombination frequency of chromosomal genes.

Our findings nonetheless mirror an earlier report that protoplast fusion resulted in an apparently high frequency transfer of the actinomycin biosynthesis determinant in several *Streptomyces* sp.[80]. The reason for this was not established.

The results of our conjugal mating experiments are not directly comparable with those from the protoplast fusion experiments because, in these two types of genetic crosses with *Streptomyces*, the mechanism of DNA transfer and the characteristics of DNA recombination events are believed to be quite different[17]. The data in Table 3 show, nevertheless, that infectious transfer of the Las$^+$ characteristic did not take place for the few strains tested. Yet we realize that these data may not be convincing evidence against infectious transfer of the *las* genes since an extensive linkage map and different fertility variants were required to demonstrate infectious transfer of SCP1[18].

The frequent loss and restoration of antibiotic production by *S. lasaliensis* demonstrated here is apparently not due to deletion of large DNA segments, their frequent amplification, or the presence of *las* genes on the pKSL plasmid. We therefore suggest that future work should examine whether another mobile genetic element (*e.g.*, a plasmid, a transposon, or an insertion sequence) is controlling antibiotic production in *S. lasaliensis*.

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