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Bacillus subtilis Mutant Deficient in the Ability To Produce the Dipeptide Antibiotic Bacilysin: Isolation and Mapping of the Mutation

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A *Bacillus subtilis* mutant which carries a lesion in a gene specific to the synthesis of the dipeptide antibiotic bacilysin was isolated. A derivative strain in which transposon Tn*917* had inserted near the bacilysin lesion was isolated and used as the donor in PBS-1 transduction mapping experiments. The *bac-1* locus was mapped between the *ctrA* and *sacA* loci, near 90% on the standard *B. subtilis* 168 chromosome map.

We are interested in the antibiotic bacilysin as a model for studies on secondary metabolism because of its simple dipeptide structure (9) and its production by the well-characterized strain of *Bacillus subtilis* designated Marburg or 168 (3, 9). It was previously shown that L-alanine is incorporated directly during bacilysin biogenesis (7) and that anticapsin, the second amino acid moiety of bacilysin, is derived from the aromatic amino acid pathway (9), branching from prephenate (3). A nonribosomal bacilysin synthetase (amino acid ligase) which can form bacilysin from its constituent amino acids in vitro has been detected in cell extracts of *B. subtilis* (8).

We would ultimately like to understand the biology of bacilysin biosynthesis at the molecular level. As a first step, we isolated a new mutant derivative of *B. subtilis* 168 which has lost the ability to synthesize bacilysin. We subsequently isolated a strain with transposon Tn*917* inserted in the chromosome near the location of the bacilysin lesion. The bacilysin locus was mapped indirectly by PBS-1 transduction of the linked transposon.

Mutagenesis and screening. Nitrosoguanidine-generated mutants of strain PY79, generated by standard methods (5), were plated directly on the surface of assay medium which had been seeded with approximately 7×10^7 CFU of *Staphylococcus aureus* ATCC 9144, an indicator strain sensitive to bacilysin, per ml. About 90% of the colonies initially picked as zone free formed a zone on repeat plates. Approximately 50 to 100 isolates remained zone free on repeat assay plates. These isolates were inoculated into the production broth of Perry and Abraham (6) and grown in liquid culture under standardized conditions. The culture broth was sampled at several times during exponential growth and the stationary phase and assayed for bacilysin titer. One isolate, designated ng79, never accumulated any detectable bacilysin (Bac^- phenotype; data not shown).

Characterization of mutant ng79. We previously showed that lesions in the aromatic amino acid pathway result in a Bac^- phenotype (3). The Bac^- strain ng79 was found to be prototrophic and was able to grow on minimal medium with glucose or sucrose as the sole carbon source. Strain ng79 was found to form spores as judged by phase-contrast microscopy and by continued viability after exposure to a temperature of 90°C for 10 min. The growth rate of the mutant was similar to that of the parent strain PY79 under the standardized conditions routinely used for bacilysin production. Culture broth supernatants were assayed for bacilysin titer, and cell extracts were assayed for bacilysin synthetase activity (Table 1). While the *aroA* strain 1A130 failed to accumulate bacilysin in the culture broth, it had a level of bacilysin synthetase activity similar to that of the producing strain, PY79. This was previously observed and was interpreted as support for the conclusion that an aromatic acid pathway intermediate (prephenate) is a precursor to the anticapsin moiety of bacilysin (3). The new mutant, ng79, had neither a detectable bacilysin titer in culture broth nor a detectable bacilysin synthetase activity.

Construction of strains to map the *bac-1* locus. The Bac^- phenotype is not a selectable marker, and we found scoring it to be difficult and often impossible in the backgrounds of many of the auxotrophic markers of the common genetic mapping strains. To circumvent this problem and prepare for future research, we chose to map the site of the lesion responsible for the Bac^- phenotype of strain ng79 (*bac-1* locus) indirectly by its proximity to a selectable marker. We used the transposon Tn*917* as a selectable marker (Tn*917* carries an *erm* gene which imparts resistance to the macrolide-lincosamide-streptogramin B antibiotics [MLS^r]) which could, theoretically, be found near any locus on the chromosome.

We took advantage of the efficient homologous recombination characteristics of competent *B. subtilis* cells in identifying a Tn*917* insertion near the *bac-1* locus by looking for cotransformation of strain ng79 to MLS^r and to the Bac^+ phenotype. At the low concentrations of DNA used in these transformations, the event most likely to be responsible for cotransformation would be a double-crossover recombination event which would introduce a transposon, previously inserted near the *bac-1* locus, into the ng79 chromosome and simultaneously repair the *bac-1* lesion. Donor DNA was isolated by the method of Errington (2) from a pool of *B. subtilis* cells which had been mutagenized with Tn*917* and propagated without intentional selection (the cells were a generous gift of K. Sandman and P. Youngman, Harvard

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University, Cambridge, Mass.). Of approximately 3,000 MLS^r transformants examined, one colony formed a zone of inhibition in the bacilysin colony assay. This isolate (strain MH18) was reexamined and found to be fully capable of bacilysin production (data not shown). Linkage of Tn*917* to the *bac-1* locus was confirmed (see below).

In preparation for transduction mapping of Tn*917*, we made use of the marker replacement technique described by Youngman et al. (10) to substitute a *cat* (chloramphenicol acetyltransferase) gene for the *erm* gene because we found it easier to work with chloramphenicol resistance (Cm^r) than with MLS^r. Strain MH18 was transformed to Cm^r with plasmid pTV21Δ2 to generate strain MH21 and with plasmid pTV20 to generate strain MH20. Transformation of strain ng79 was used to confirm the loss of MLS^r upon transformation with pTV21Δ2 and to confirm linkage of the *cat* gene of both MH20 (100 of 100 Cm^r colonies were MLS^r; 19 of 100 Cm^r colonies were Bac$^+$) and MH21 (0 of 96 Cm^r colonies were MLS^r; 14 of 96 Cm^r colonies were Bac$^+$) to the bacilysin lesion. In addition, strain MH22 with the phenotype Cm^r MLS^r Bac$^-$ was isolated, presumably as a result of crossover between the Tn*917* insertion site and the *bac-1* locus.

Genetic mapping of *bac-1* locus. Strain MH20 was used to generate PBS-1 donor phage for transduction mapping (4) of the strains in the Dedonder kit (1). The only markers to which the *cat* gene was found to be linked were the *ctrA* and *sacA* genes of kit strain QB123; it was linked with a cotransductional frequency of about 65% to each marker. Table 2 shows the results of three-factor analyses which established that the Tn*917* insertion occurred between the *sacA* and *ctrA* genes, located at approximately 90% on the standard *B. subtilis* chromosome map (11). Attempts to order the *bac-1* locus relative to these other three markers by using ng79 and MH22 as sources of phage donor were inconclusive, in part because only *ctr*$^+$ transductants could be scored for Bac phenotype (both ammonia and cytidine interfere with bacilysin production) and in part because of difficulties in scoring the bacilysin phenotype. While we found it easy to judge a few Bac$^+$ colonies in a Bac$^-$ background, we found it difficult to reliably identify Bac$^-$ colonies in a predominantly Bac$^+$ background. Thus, while we were not able to position the *bac-1* locus relative to the Tn*917* insertion, we were able to confirm their proximity to one another. When ng79 was donor to QB123, 26 of 26 *sac*$^+$ *ctr*$^+$ transductants were Bac$^-$, and when MH22 was donor to QB123, 20 of 20 Cm^r *sac*$^+$ *ctr*$^+$ transductants were also Bac$^-$. Ordering of the *bac-1* locus relative to the Tn*917* insertion site of strain MH18 would require higher-resolution mapping with additional markers.

No other mutations related to antibiotic biosynthesis have been reported to be in this region of the chromosome. However, the *spo0F* locus and two partial supressors of sporulation (*rev-4* and *abrA*) are located around 89% (reviewed in reference 11). We do not know if any of these loci are related to the *bac-1* locus. Mutants with lesions in the aromatic acid pathway, which are deficient in a precursor to bacilysin, retain bacilysin synthetase activity in the absence of active bacilysin synthesis (3). Since the Bac$^-$ strain ng79 is Aro$^+$ and has lost cell-free bacilysin synthetase activity, the *bac-1* mutation may be a lesion in the synthetase structural gene. The regulation of bacilysin genes is unknown, so the mutation may alternatively be due to a lesion in a regulatory locus. However, no pleiotropy was detected for the ng79 mutant, so whether a regulatory or structural locus was affected, the mutation appears to be specific to bacilysin synthesis. The *bac-1* lesion could be mapped to a single chromosomal region, so we do not believe the phenotype to be due to lesions of multiple genes unless they are in a cluster.

TABLE 1. Analysis of culture broths for bacilysin titer and of cell extracts for bacilysin synthetase activity

Strain	Colony assay phenotype	Bacilysin titer (U/ml)[a]	Bacilysin synthetase sp act (U/mg of protein per 5 min)[a,b]
PY79	Bac$^+$	39	360
1A130	Aro$^-$ Bac$^-$	<1.5	400
ng79	Bac$^-$	<1.5	<20

[a] Bacilysin units are as defined in reference 3.
[b] Assayed by the method of Sakajoh et al. (8).

TABLE 2. Three-factor analysis of PBS-1 transduction of QB123 (*ctrA1 sacA321 trpC2*) with donor phage prepared from strain MH22 (Cm^r MLS^r *bac-1*)

Selection[a]	Cotransduction	Frequency (%)
Sac$^+$	Ctr$^+$ Cm^r	52/105 (40)
	Ctr$^+$ Cm^s	0/105 (0)
	Ctr$^-$ Cm^r	24/105 (23)
	Ctr$^-$ Cm^s	39/105 (37)
Ctr$^+$	Sac$^+$ Cm^r	10/54 (19)
	Sac$^+$ Cm^s	0/54 (0)
	Sac$^-$ Cm^r	23/54 (43)
	Sac$^-$ Cm^s	21/54 (39)
Cm^r	Sac$^+$Ctr$^+$	21/53 (40)
	Sac$^+$Ctr$^-$	2/53 (4)
	Sac$^-$Ctr$^+$	23/53 (43)
	Sac$^-$Ctr$^-$	7/53 (13)

[a] The Sac and Ctr phenotypes were selected as described in reference 1.

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