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# Identification of a Streptogramin A Acetyltransferase Gene in the Chromosome of *Yersinia enterocolitica*

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**Streptogramins are polypeptide antibiotics inhibiting protein synthesis by the prokaryotic ribosome. Gram-positive organisms are susceptible to streptogramins, while most gram-negative bacteria are intrinsically resistant. We have found a genomic fragment from a *Yersinia enterocolitica* isolate with an open reading frame coding for a polypeptide similar to the virginiamycin acetyltransferases found in various plasmids from gram-positive bacteria. The susceptible *Escherichia coli* strain DB10 was transformed to resistance to the type A streptogramins and to mixed (A + B) streptogramins upon introduction of a plasmid containing that gene. In addition, we showed streptogramin acetylating activity in vitro dependent on the presence of the *Y. enterocolitica sat* gene. Southern blot hybridization experiments showed that the *sat* gene was present in all the *Y. enterocolitica* isolates examined. These data together show that the gene in the *Y. enterocolitica* chromosome encoded an active streptogramin acetyltransferase. The deduced sequence of the *Y. enterocolitica* Sat protein was close to those of *sat* gene products found in gram-positive bacteria and cyanobacteria, suggesting a common evolutionary origin.**

Streptogramins are a group of cyclic peptide antibiotics produced by some *Streptomycetes*. They are divided into two classes, A and B, according to chemical structure (9). Both A and B streptogramins act to block protein synthesis by inhibition of the peptidyl transferase domain in the 50S subunit of the prokaryotic ribosome. Compounds of the A and B classes act synergistically. This property makes the A + B mixtures active against many bacterial pathogens (20). The poor solubility in water of these antibiotics has reduced their use in human medicine. However, some streptogramins (virginiamycin) have been used as animal feed additives. Recently, new streptogramins have been developed with increased water solubility and hence better pharmacological properties (7). The quinupristin-dalfopristin combination has proved useful in fighting infections produced by dangerous antibiotic-resistant strains of staphylococci or enterococci (14, 16, 22). Resistance to the class A streptogramins takes place either by active efflux mediated by the gene *vga* in staphylococci (3) or by inactivation via enzymatic acetylation of the antibiotic (12). This inactivation is catalyzed by streptogramin (virginiamycin) acetyltransferases, the products of *sat* or *vat* genes (1, 5, 23). These are small (24-kDa) enzymes, closely related to each other, and the sequences of their carboxyl-terminal halves are very similar to those of other acetyltransferases (5). Resistance to class B streptogramins can be obtained by efflux, modification of the drug, or rRNA methylation, which also results in resistance to macrolides and lincosamide (18). Due to the interplay of A and B streptogramins, resistance to the A compounds usually results in resistance to the mixture.

Gram-negative organisms are intrinsically resistant to the streptogramins. Since their ribosomes are sensitive to inhibition, it was thought that the mechanism of this intrinsic resistance was the exclusion of the antibiotics from the cytoplasm (15). Streptogramins are poorly hydrophilic molecules whose molecular size is greater than 500 Da. The gram-negative outer membrane constitutes a strong permeability barrier to streptogramin entrance into the periplasm. No carrier for streptogramins through the inner membrane is known. The hydrophobic pathway could be the main route for streptogramins into the bacterial cytoplasm. Organisms of the genus *Neisseria* and *Haemophilus* are more permeable to larger hydrophilic molecules, and consequently they can be susceptible to streptogramins (28). In addition to the permeability-related intrinsic resistance, resistance to class B streptogramins in *Escherichia coli* and other enterobacteria can also be mediated by the *ermB* gene, probably acquired from gram-positive organisms (18). Resistance to streptogramins of the A group by mechanisms other than the permeability barrier has never been reported, either in *Yersinia* spp. or in other enterobacteria.

Here we describe the presence of a gene encoding streptogramin A acetyltransferase activity in the chromosome of a *Yersinia enterocolitica* isolate. The possible role of this gene in the evolution of bacterial resistance to streptogramins is also discussed.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The following *Y. enterocolitica* strains were used in this work: P1403 and P219 (biotype 1A); WA and 1354 (biotype 1B); My79b and IP97 (biotype 2); and IP4124, IP22273, IP22274, H6, H14, Y56, and Y60 (biotype 4). Most of the strains were from our laboratory collection and have already been described (11). Strains IP4124, IP22273, and IP22274 were obtained from Jeanette N. Pham. The *E. coli* strain DB10, a fusidic acid-susceptible derivative of *E. coli* PR7 (10), and DB11, another *E. coli* mutant susceptible to streptogramins (1), were used as recipients for recombinant plasmids. *Staphylococcus aureus* BM3002 and *Enterococcus faecium* BM4145 (obtained from P. Courvalin) were used as control streptogramin A-resistant strains known to produce streptogramin A acetyltransferases. *S. aureus* ATCC 28213 was used as a control susceptible strain in MIC determinations. To clone genomic DNA fragments, we used the high-copy-number plasmid vector pK18 (21), which contains a kanamycin resistance selection marker.

**Antibiotics.** Virginiamycin M1 (a class A streptogramin) and polymyxin B nonapeptide were purchased from Sigma. Dalfopristin (RP54476, another class A streptogramin), quinupristin (RP57669, a class B streptogramin), and a 70:30 mixture of the latter two compounds (Synercid, also referred to as RP59500), were the generous gifts of Rhône Poulenc Rorer Laboratories.

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**Genetic and DNA methods.** Bacterial transformation, DNA purification, cloning, and DNA sequencing were carried out by standard methods basically as described in reference 24.

**Analysis of the DNA sequence.** The deduced sequence of the *Yersinia sat* gene product was compared against nucleotide sequences in the nonredundant (GenBank, EMBL, DDBJ, and PDB) databases, using the program TBLASTN 2.0.9 (6) in the Blastserver at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/BLAST/). Preliminary sequence data from unfinished genomes were obtained from the website of The Institute for Genomic Research (TIGR) (http://www.tigr.org/). Multiple sequence comparisons and tree construction were performed with the PILEUP program from the Genetics Computer Group (GCG), version 10, package (13), in the National node of the European Molecular Biology Network in the Centro Nacional de Biotecnología (EMBNET/CNB), Madrid, Spain, using its website (http://www.es.embnet.org/).

**Southern blot hybridization of *Y. enterocolitica* chromosomes.** Chromosomal DNA from stationary-phase cultures of the different strains was purified after lysis with guanidine thiocyanate as described elsewhere (17), digested with the restriction endonucleases *Eco*RI and *Hin*dIII, separated in a 0.8% agarose gel, and transferred to positively charged nylon membranes (Boehringer Mannheim). A 624-bp *Eco*RV/*Xba*I fragment containing the *sat* gene from the Y56 chromosome was labeled with digoxigenin and used to probe the *Yersinia* chromosomes under stringent conditions (42°C in 50% formamide), including a final high-stringency wash at 68°C in 1:10-diluted SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate. Blots were developed using a chemiluminescence detection kit from Roche.

**MIC determinations.** MICs were determined on Mueller-Hinton agar plates containing doubling concentrations of antibiotics between 1 and 256 μg $ml^{-1}$. MICs were also determined in the presence of 3 μg of the membrane permeabilizing agent polymyxin B nonapeptide $ml^{-1}$ (27). Inocula of $10^4$ CFU were spotted onto the plate.

**Determination of streptogramin A acetyltransferase activity.** In vitro streptogramin A acetyltransferase activity was determined spectrophotometrically, essentially as described previously (23, 26). Crude enzyme extracts were prepared from late-stationary-phase cells by sonic disruption in Tris (50 mM) (pH 7.8) buffer containing 50 μM β-mercaptoethanol. Crude extracts were clarified by centrifugation at 20,000 × *g* for 20 min at 4°C, and supernatants were directly used in enzymatic reactions. The assay mixture contained 0.1 mM streptogramin A, 0.1 mM acetyl coenzyme A, and 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Protein concentrations in extracts were determined by the method of Bradford (8) using a commercial reagent (Bio-Rad).

**Nucleotide sequence accession number.** The nucleotide sequence of the *satA* gene from *Y. enterocolitica* Y56 has been deposited in the GenBank Data Library with the accession number AF170730.

## RESULTS

**Nucleotide sequence of the *Y. enterocolitica sat* gene: sequence analysis and comparison.** While characterizing the genome region of *Y. enterocolitica* Y56 around the *blaA* gene (25), we obtained a 1.5-kb *Eco*RI fragment which was cloned in the *E. coli* kanamycin resistance vector pK18. This plasmid was called pAS3. The 1.5-kb *Eco*RI DNA fragment was further subcloned using *Xba*I and *Eco*RV restriction sites, and its complete nucleotide sequence was determined on the two DNA strands, using universal and specific DNA primers. The G+C content of the sequence was 49.4%. This value is similar to the overall G+C content of *Y. enterocolitica* DNA (48.5% ± 1.5%). A 663-bp open reading frame (ORF) was found in the sequence. The translated product from this ORF (221 amino acids; 24.4 kDa) was used to investigate the presence of homologous genes in the nonredundant nucleotide sequence database using the TBLASTN program. We found that the gene was homologous with streptogramin A acetyltransferases from *Synechocystis* spp., *S. aureus*, and *E. faecium*. Comparison was also carried out with the nucleotide sequence of unfinished genomes kindly provided by TIGR. Two additional homologous genes were found in the genomes of *Shewanella putrefaciens* and *Pasteurella multocida*. The deduced amino acid sequence of the *Y. enterocolitica* ORF was aligned with those of the virginiamycin acetyltransferases from the other bacteria (Fig. 1). The evolutionary distances among the aligned sequences were calculated and used to construct the phylogenetic tree shown in Fig. 2. Based on the high degree of similarity among the proteins, we assumed that the *Y. enterocolitica* gene encoded a streptogramin A acetyltransferase and called it *sat*.

```
          1                                                  50
  yersat  MEDKPILGPD PQCKHPMVGF SQVCFIKNTT QN.PNIIIGD YTYYDDPQDS
  synvat     MY--- --N----A-- A-I------V S-.-------- -------E--
staphvata -NLNNDH--- -ENIL-IK-N RNLQ---P-I T-.E--LV-E -S---SKRG.
staphvatb    MKY--- -NSIY-HEEI KS-------I T-.----V-- ----S-VNGA
 cohnivat -KWQNQQ--N -EEIY-IE-N KH-Q---PSI TK.---LV-E -S---SKDG.
shewavat      --N -ND-A-LK-- P--G-V--FI TR.N---V-- -------AGP
multovat       -- KDQIF-IKHY HRLC-L--LV RH.----V-- F-----AENV
  entvat    M--N -MKMY-IE-N KS-Q---PIL EKLE-VEV-E -S---SKNG.

          51                                                 100
  yersat  ENFERNVLYH YPFIGDKLII GKFCALAHGV KFIMNGANHK MSGLSTYPFN
  synvat  ---------- F--V--R--- -------RN- ---------A -E-F-----A
staphvata -S--DQ---- -EV------- -R--SIGP-T T--------R -D-.-----H
staphvatb -K--EH-TH- -E-R----V- -----I-E-I E--------R -NSIT-----
 cohnivat -S--SQ---- -EL------L ----SIGP-T T--------R -D-.--F---
shewavat -R--S----- FD-V------ -----I-KN- ---------S V--F-----Y
multovat ----K--K-- FD-L------ ----MIGSD- ---------Q -NAI-----G
  entvat -T-DKQI--- --ILN---K- ----SIGP-- TI--------R -D-.------

          101                                                150
  yersat  IFGNGWERVA PSRDELPYKG DTHVGNDVWI GYDVLIMPGV TIGNGAIISS
  synvat  --A---Q.T- ELEE-P-H-- --VI------ --EAV----I KV-D----AA
staphvata L-RM---KYM --LKD--L-- -IEI------ -R--T----- K--D----AA
staphvatb -M-----KAT --LED--F-- --V------- -QN-TV---I Q--D---VAA
 cohnivat L------KHT -TLED----- N-EI------ -R--T----- K-------AA
shewavat -------KAT -KPED--F-- --RI-H---- --NAT----- N--H----A-
multovat ---YE-AKAL -EKA--SS-- --VI-NE--- --NAT----- K--D----GT
  entvat L------KHM -KL-Q--I-- --II------ -K--V----- K--D---VAA

          151                                                200
  yersat  RSVVTRDVPA YSVVGGNPAT LIKNRFSAEV IGKLQTIAWW DWPIDAISRN
  synvat  KA---NH-AP -TI------R P-RQ--KD-- VQA-L----- --D-EK-T--
staphvata EA---KN-AP --I-----LK F-RK---DG- -EEWLALQ-- NLDMKI-NE-
staphvatb N----K---P -RII----SR I--K--ED-L -DY-LQ-K-- --SAQK-FS-
 cohnivat K----KN-DP --------SR ---I---K-K -AA-LKVR-- -LE-ET-NE-
shewavat Q----K---P -A-------- I--L--EQT- -DE-LA---- ----EK-TEH
multovat N----KN--P -AI-A----R V-RK---P-K -EQ-LALK-- --E-EK-T--
  entvat N---VK-IAP -MLA-----N E--Q--DQDT -NQ-LD-K-- N----I-NE-

          201                223
  yersat  LHLIV.AGDI EALARAASEI DHT
  synvat  -DK-A.GA-- ---KNCS
staphvata -PF-I.N--- -M-K-KRKLL -D-
staphvatb -ETLC.SS-L -KIKS
 cohnivat IDC-L.N--- KKVK
shewavat --A--.SA-L SQ-
multovat -DFLT.G
  entvat IDK-LDNSI- REVIWKK
```

FIG. 1. Multiple alignment of Vat proteins from different microorganisms. Amino acids are represented by the one-letter standard code. Gaps are represented by dots. A dash indicates that the residue in that position is the same as that in the *Y. enterocolitica* sequence shown in the top line. Residues conserved in all the sequences are boldfaced. Abbreviations: yersat, *Y. enterocolitica* Y56 Sat (GenBank accession number AF170730); synvat, Vat of *Synechocystis* spp. (D13960); staphvata, *S. aureus* VatA (L07778); staphvatb, *S. aureus* VatB (U19459); cohnivat, *S. cohnii* VatC (AF015628); shewavat, *S. putrefaciens* putative Sat; multovat, *P. multocida* PM70 putative Sat; entvat, *E. faecium* SatA (L12033). Preliminary sequence data for *S. putrefaciens* and *P. multocida* PM70 were obtained from the website of The Institute for Genomic Research (http://www.tigr.org/).

**Contribution of the *sat* gene to streptogramin resistance in *E. coli* and *Y. enterocolitica*.** To determine the contribution of the *sat* gene to the resistance to streptogramins in *E. coli*, we used the susceptible strains DB10 and DB11. The MICs of a class A streptogramin (dalfopristin), a class B streptogramin (quinupristin), and the 70:30 dalfopristin-quinupristin mixture, were determined for the *E. coli* strains with and without plasmids and for a collection of *Y. enterocolitica* strains representative of the different biotypes (data not shown). Upon introduction of the *sat* gene-containing plasmid pAS3 in the *E. coli* strains, the MIC of the class A streptogramin increased from 2 to 8 μg $ml^{-1}$ for *E. coli* DB10 and from 4 to 32 μg $ml^{-1}$ for *E. coli* DB11. The MIC for the *Y. enterocolitica* strain Y56 was 64 μg $ml^{-1}$. Detailed results of these experiments are summarized in Table 1.

**In vitro activity of streptogramin acetyltransferase.** The activity of the *Y. enterocolitica sat* gene was further investigated in vitro by determination of the acetylating activity in crude protein extracts from *Y. enterocolitica* and *E. coli* with and without the *sat* plasmid pAS3 on class A streptogramins. We used two

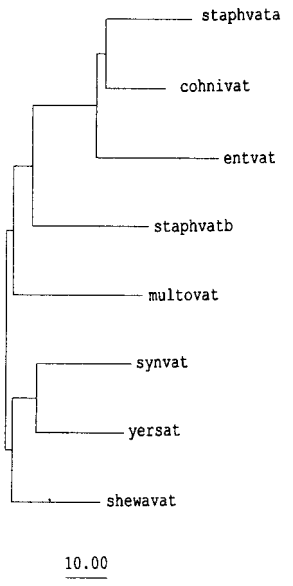


FIG. 2. Phylogenetic tree showing the evolutionary relationship of the *Y. enterocolitica sat* gene product with other streptogramin acetyltransferases. The tree was elaborated with the program GROWTHTREE from the GCG package (13) from a distance matrix calculated from the alignment shown in Fig. 1. The length of each branch is proportional to the number of substitutions per 100 residues. Bar, 10 substitutions per 100 amino acids. Abbreviations are as explained in the legend to Fig. 1.

different substrates for this reaction, namely, virginiamycin M1 and dalfopristin. Extracts from *Y. enterocolitica* Y56 and *E. coli* DB10 containing plasmid pAS3 showed acetylating activity on the two class A streptogramins. The activity of the extracts from *E. coli* DB10 containing only the vector pK18 was negligible (Table 2).

**Investigation of the presence of *sat* genes in chromosomes from other *Y. enterocolitica* isolates.** To determine whether the presence of the *sat* gene was a peculiarity of the *Y. enterocolitica* strain Y56 or, on the contrary, was commonplace in the chromosome of *Y. enterocolitica*, we probed the chromosomes of a collection of *Y. enterocolitica* strains with the *sat* gene probe. All the strains tested hybridized with the probe under high-stringency conditions, indicating that they contained at least one copy of the gene (Fig. 3). We observed basically three different sizes of hybridizing bands among the different strains, which correlated with their biotype. Biotype 1A strains showed a band of about 4 kb, strains of biotypes 1B and 2 showed a band of 3.4 kb, and biotype 4 strains showed a band of 1.5 kb. A similar result had been obtained previously when the same strains were probed for the presence of *bla* genes (11); these results probably reflect differences in genomic organization in the different *Y. enterocolitica* biotypes. The *sat*-containing bands in the biotype 4 strains showed small but noticeable size differences. The band from Y56 (which is the same 1.5-kb *Eco*RI fragment cloned in plasmid pAS3) was about 100 bp larger than the bands from strains IP22274 and H14. The reason for this size polymorphism is not understood at the moment. A fainter, smaller hybridization band was also observed in most strains. The nature of this second hybridization band is also unknown, but it could be due to the presence of an additional acetyltransferase gene in the *Y. enterocolitica* chromosome.

TABLE 1. MICs of streptogramins for *E. coli* strains with and without plasmid pAS-3 and for *Y. enterocolitica* Y56

| Strain | MIC (μg ml$^{-1}$) of[a]: | | |
|---|---|---|---|
| | A | B | A + B |
| *E. coli* | | | |
| DB10 | 2 | 64 | 2 |
| DB10 (pK18) | 2 | 64 | 2 |
| DB10 (pAS3) | 8 | 256 | 8 |
| DB11 | 4 | 256 | 4 |
| DB11 (pK18) | 4 | 256 | 4 |
| DB11 (pAS3) | 32 | 256 | 32 |
| *E. faecium* BM4145 | 64 | 256 | 32 |
| *Y. enterocolitica* Y56 | 64 | >256 | 32 |

[a] A, the class A streptogramin dalfopristin; B, the class B streptogramin quinupristin; A + B, a 70:30 dalfopristin-quinupristin mixture.

TABLE 2. In vitro acetylating activities of crude extracts[a] on the class A streptogramins virginiamycin M1 and dalfopristin

| Extract | Activity (nmol of antibiotic acetylated/min/mg of protein) on: | |
|---|---|---|
| | Virginiamycin M1 | Dalfopristin |
| *E. coli* DB10 pK18 | 0.65 ± 0.15 | 0.46 ± 0.11 |
| *E. coli* DB10 pAS3 | 42.4 ± 7 | 31.27 ± 3 |
| *S. aureus* BM3002 | 129.7 ± 8.2 | 63.49 ± 6.4 |
| *E. faecium* BM4145 | 161.83 ± 12.3 | 82.68 ± 8.5 |
| *Y. enterocolitica* Y56 | 16.2 ± 2 | 11.06 ± 1.4 |

[a] Extracts were prepared as described in the text.

## DISCUSSION

Resistance to class A streptogramins by inactivation catalyzed by streptogramin acetyltransferase is common among gram-positive organisms, and sequences of *sat* genes from *S. aureus* (1, 5), *Staphylococcus cohnii* (2), *E. faecium* (23), etc., have been reported. In all these cases, *sat* genes were located

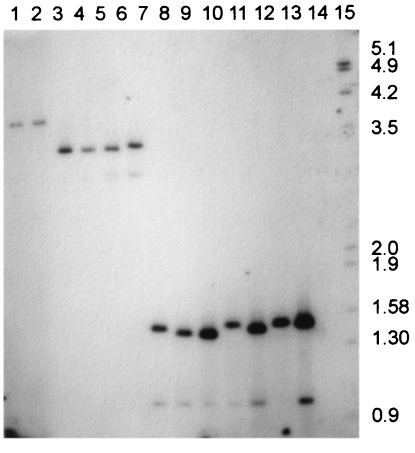


FIG. 3. Hybridization of *Y. enterocolitica* chromosomal DNA digested with *Eco*RI/*Hin*dIII with a probe containing the *sat* gene from *Y. enterocolitica* Y56. Lanes: 1, P1403; 2, P219; 3, WA; 4, 13514; 5, My79b; 6, IP97; 7, IP4124; 8, IP22273; 9, IP22274; 10, H-6; 11, H-14; 12, Y-56; 13, Y-60; 14, *E. coli* chromosomal DNA; 15, molecular mass markers. The sizes of the markers in kilobases are shown on the right.

in plasmids. Here we report the isolation of a *sat* gene from the chromosome of a *Y. enterocolitica* isolate. The gene product was first identified by sequence comparison by its high similarity with other streptogramin acetyltransferases. The sequence comparison search also revealed high similarities with the carboxyl termini of other acetyltransferases such as the chloramphenicol acetyltransferase of Tn*2424*, indicating that streptogramin acetyltransferases belong to a broad family of enzymes with different substrates, as had been previously described (5, 19).

The functionality of the *Y. enterocolitica sat* gene was demonstrated by an increase in the dalfopristin and quinupristin-dalfopristin MICs upon transfer of the *sat* gene to susceptible *E. coli* strains, and by determination of in vitro acetylating activity on the class A streptogramins dalfopristin and virginiamycin M1. The fact that the presence of this gene in a gram-negative organism has remained unnoticed until now may be due to the high level of intrinsic resistance to streptogramins in gram-negative organisms (15, 28). For most of the *Y. enterocolitica* and *E. coli* laboratory strains, the MICs of class A streptogramins were higher than 64 $\mu g\ ml^{-1}$. Since these values are much higher than the regular therapeutic levels, susceptibility testing of gram-negative organisms is considered useless and is not performed. On the other hand, the elevated intrinsic resistance to streptogramins in gram-negative bacteria devoid of *sat* genes, such as *E. coli*, raises questions about the contribution of the *sat* gene to resistance in these bacteria. How much of the resistance to class A streptogramins in *Yersinia* isolates is due to the activity of the *sat* gene? We cannot give an exact answer to this question, since we have not found any *Y. enterocolitica* strain devoid of the *sat* gene. However, we can obtain an approximate answer by use of the membrane-permeabilizing agent polymyxin B nonapeptide. The MICs of class A streptogramins for the *Yersinia* strains were reduced to 32 $\mu g\ ml^{-1}$ in the presence of this agent (data not shown). This resistance level was similar to that obtained with *E. coli* DB11 upon acquisition of the *satA*-containing plasmid pAS3. From these observations we may conclude that the elevated level of resistance observed in *Y. enterocolitica* isolates, and in most *E. coli* strains, resulted from the simultaneous action of several mechanisms, some of which still remain unknown.

We have demonstrated that the *sat* gene was present in all *Y. enterocolitica* isolates examined. This suggested that the gene is part of the bacterial genome, rather than an acquisition of a particular strain where the gene was found. Furthermore, the analysis of unfinished bacterial genomic sequences revealed that *sat* genes are often present in bacterial chromosomes of the gamma division of the purple bacteria. They were present in *P. multocida*, a close relative of *Yersinia*, as well as in *S. putrefaciens*. A *sat* gene is also present in the chromosome of the cyanobacterium *Synechocystis*. The presence of *sat* genes in all these genera, and the similarity of the product of this gene to a broad family of acetylases with many different substrates, may indicate that it plays a physiological role consisting in the acetylation of some unidentified substrate and that streptogramin acetylation is an undesired collateral effect. Furthermore, the chromosomal location of this gene contrasted with the plasmid location of *sat* genes in gram-positive bacteria, suggesting that plasmid genes could have originated from their chromosomal counterparts (or vice versa) after a process of gene mobilization and transfer, well documented in many of these organisms. This hypothesis could also be supported by the level of identity found between the deduced sequences of Sat proteins from gram-positive and gram-negative bacteria.

In addition to their use in human medicine, some streptogramins (virginiamycin) are used as growth promoters in farm animals. It has been reported that this practice resulted in the selection of staphylococci and enterococci resistant to virginiamycin and to other streptogramins in poultry (29). *Y. enterocolitica* is a pathogen closely associated with pigs, one of the animal species fed virginiamycin. This association may have selected for an acetylase gene with a higher affinity for streptogramins, capable of conferring resistance to these drugs. While the finding of *sat* genes in *Y. enterocolitica* can be of little relevance to the susceptibility of this species to streptogramins, due to its high level of intrinsic resistance, it can be very important from an epidemiological point of view, since we may have identified a reservoir of *sat* genes ready to be mobilized to other human pathogens whose resistance to streptogramins may represent a very serious clinical problem.

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