How Does Pseudomonas fluorescens Avoid Suicide from Its Antibiotic Pseudomonic Acid?

EVIDENCE FOR TWO EVOLUTIONARILY DISTINCT ISOLEUCYL-tRNA SYNTHETASES CONFERRING SELF-DEFENSE*

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Two iso-leucyl-tRNA synthetases (IleRSs) encoded by two distinct genes (ileS1 and ileS2) were identified in pseudomonic acid (mupirocin)-producing Pseudomonas fluorescens. The most striking difference between the two IleRSs (IleRS-R1 and IleRS-R2) is the difference in their abilities to resist pseudomonic acid. Purified IleRS-R2 showed no sensitivity to pseudomonic acid even at a concentration of 5 mM, 10^3 times higher than the Ki value of IleRS-R1. The amino acid sequence of IleRS-R2 exhibits eukaryotic features that are originally found in eukaryotic proteins. Escherichia coli cells transformed with the ileS2 gene exerted pseudomonic acid resistance more than did those transformed with ileS1. Cells transformed with both genes became almost as resistant as P. fluorescens. These results suggest that the presence of IleRS-R2 could be the major reason why P. fluorescens is intrinsically resistant to the antibiotic. Here we suggest that the evolutionary scenario of the eukaryotic ileS2 gene can be explained by gene acquisition and that the pseudomonic acid producer may have maintained the ileS2 gene to protect itself from pseudomonic acid.

Pseudomonic acid (mupirocin) is an antibacterial agent produced by Pseudomonas fluorescens NCIB10586 (1). This antibiotic is known as a potent inhibitor of many bacterial iso-leucyl-tRNA synthetases (IleRSs).1 It competitively inhibits IleRS with respect to isoleucine (2), thereby arresting protein synthesis. The inhibitory constants (Ki) for prokaryotic (eubacterial and archaebacterial) IleRSs in in vitro aminoacylation reaction are generally 10^-8 to 10^-9 M (2–4). Meanwhile, this antibiotic has little or no potency toward eukaryotic IleRSs (2, 5). This striking specificity in the mode of action and in its unique structure place pseudomonic acid into an unusual class of antibiotic agents (6, 7) and have led to the clinical use of pseudomonic acid as a topical antibacterial agent to prevent Staphylococcus aureus infection (8, 9).

IleRS, the target of antibiotic pseudomonic acid, is one of the aminoacyl-tRNA synthetases (aaRSs), which play a crucial role in the first step of protein biosynthesis, because they are responsible for accurate charging of specific tRNAs with their cognate amino acids (10, 11). Twenty amino acid-specific aaRSs can be divided into two classes, each of which contains 10 enzymes (11, 13, 14). Class I aaRSs, including IleRS, each display two short common consensus sequences, called HIGH and KMSKS, which form a Rossmann nucleotide binding fold and play an important role in enzyme catalysis. Furthermore, based on their species-specific conserved sequences, aaRSs can generally be subdivided into either a eubacteria type or an archaeal/eukaryote type (12).

Like other general antibiotic-producing organisms, P. fluorescens is entirely insensitive to the antibiotic it produces, pseudomonic acid (18). Antibiotic-producing bacteria have several common strategies to prevent their own antibiotics from killing them (19). By kinetic analysis it has been revealed that the affinity of P. fluorescens crude IleRS for pseudomonic acid is dramatically lower than that of Escherichia coli (2, 18). It has thus been considered that the difference in the molecular structure of IleRS causes difference in affinity for the antibiotic (the difference in inhibition) and that this structural alteration makes P. fluorescens cells highly resistant to pseudomonic acid.

In this report we provide evidence for two distinct IleRSs (IleRS-R1 and IleRS-R2) in P. fluorescens. The former is an enzyme that we reported earlier (25), and the latter is a quite newly found enzyme that has a markedly reduced affinity for the antibiotic and thereby confers upon the organism a strong resistance to pseudomonic acid. We concluded that the self-defense of the pseudomonic acid producer might be caused mainly by the production of an additional IleRS. The amino acid sequence deduced from the novel ileS gene was similar to eukaryotic IleRSs and evolutionarily distinct from IleRS-R1. The physiological significance of two ileS genes in pseudomonic acid-producing P. fluorescens, and the origin of the newfound ileS gene is also discussed.

**EXPERIMENTAL PROCEDURES**

Materials, Enzymes, and Chemicals—Biochemical and molecular biological procedures were performed using commercially available enzymes, chemicals, and other materials. The pseudomonic acid was a gift from SmithKline Beecham Pharmaceuticals (Betchworth, Surrey, UK).

Culture Media and Growth Conditions—E. coli cells were grown in LB or SOB medium (45). P. fluorescens cells were grown at 26 °C in either LB culture medium or in agar medium (pH 7.0), the latter containing 10 g of Bacto-tryptone, 10 g of Bacto-proteose peptone number 3, 1.5 g of dipotassium phosphate, and 1.5 g of magnesium sulfate (per liter).

Enzyme Assay of IleRS and Kinetic Studies—Aminoacylation activi-
ties were measured in the standard mixture (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 40 mM KCl, 50 µM ZnCl₂, 1 or 4 mM ATP, 5 mg/ml E. coli tRNA, and appropriate amounts of IleRS). Kinetic constants were determined as described previously (25).

Preparation of Crude Cell Extract—P. fluorescens cells were cultured in LB medium (1 liter) at 26 °C for 12 h. Cells were harvested by centrifugation at 12,000 × g for 10 min. The cell pellets were suspended in buffer A1 (20 mM potassium phosphate (pH 7.0), 10% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). The suspensions were sonicated, and then the cell debris was pelleted by centrifugation at 10,000 × g for 10 min.

Activity Separation of the Two IleRS of P. fluorescens by DEAE-Sephacl Chromatography—P. fluorescens NCIB10586 cells were cultured in LB medium (1 liter) at 26 °C for 12 h. Cells were harvested by centrifugation at 10,000 × g for 10 min. The supernatant was ultra centrifuged at 165,000 × g for 2 h and then dialyzed overnight against buffer A5. The crude enzyme was used for kinetic analysis. The protein concentrations were determined by the method of Lowry (see Ref. 46).

Cloning and Sequencing of P. fluorescens ileS2 Gene—For cloning, EcoRI-digested P. fluorescens DNA was fractionated by agarose gel electrophoresis. An 8.5-kb fragment of DNA was identified by Southern blot analysis and electroeluted from the gel. This fragment was cloned into the E. coli expression vector pUC118. The plasmid DNA was fractionated by electrophoresis through an SDS-10% polyacrylamide gel and transferred onto an Immobilon membrane. The protein of interest was detected using specific antibodies and visualized by the ECL system (Amersham Biosciences).

Plasmid Construction and Expression of IleS2 in E. coli—The construction of plasmid pEXR1 (the former name is pPFB7) was described previously (25). To construct pEXR2, the P. fluorescens ileS2 gene with PCR-modified 5′- and 3′-ends was ligated into the NdeI/NotI double-digested pUC18 vector. The primers used were 5′-GACACCGTGTTGACATGAGTCAAGGGAAAGATGTTGCCC-3′ and 5′-AAGCTTGGCAACCTTTCGAGCATTGCCTAGGCGC-3′ (for the restriction enzyme sites are underlined, and the initiation codons or stop codons on the reverse strands are in parentheses). Plasmid pR2 was used as a template. The construct was introduced into DH5α, and the ileS2 gene was expressed as described previously (25).

Overexpression and Purification of Recombinant IleRS Proteins—Recombinant IleRSs were overexpressed and purified as follows. To prepare IleRS-R1 and IleRS-R2, pEXR1 and pEXR2 were used, respectively, to transform E. coli strain TG31. Transformsants were grown in 3 liters of SOD medium containing ampicillin (50 µg/ml) and isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) at 30 °C for 10 h with shaking. Cells were harvested and suspended in buffer A1 containing 2 µg/ml antipine, 2 µg/ml leupetin, 1 µg/ml pepstatin, and 1 µg/ml chymostatin. The cells were broken by sonication, and crude extracts were centrifuged at 30,000 × g for 20 min. The supernatants were subjected to ultracentrifugation at 100,000 × g for 6 h to remove cell debris and ribosomes. Purified IleRS proteins were obtained by three steps of column chromatography (DEAE-Toyopearl (or DEAE-Sephacl), n-butyly-Toyopearl, and hydroxyapatite) as in the case of the purification of native IleRS-R2 from P. fluorescens. Active fractions were pooled, concentrated, and dialyzed against buffer B. Purified IleRS-R1 and IleRS-R2 were used for preparing polyclonal antibodies. The enzyme activity of recombinant IleRS-R1 and IleRS-R2 expressed in E. coli cells was clearly separated from endogenous E. coli IleRS activity by column chromatography, and little or no endogenous IleRS activity can remain in these purified recombinant P. fluorescens IleRSs.

Complementation Analysis—E. coli temperature-sensitive ileS mutant Ts31 was used for complementation analysis. Ts31 transformed with ileS-containing plasmids were grown at 42 °C on LB agar plates.

In Vivo Pseudomonic Acid Resistance—Pseudomonic acid was diluted by 0.5% LB agar media (100 µl) at 10, 20, 25, 30, 40, 50, 75, 100, 150, 200, 300, 400, 500, 600, 800, and 1000 µg/ml in microtiter plates. Test organisms were grown at 30 °C overnight in the LB medium. The overnight cultures were diluted to a concentration of 0.5 × 10⁶ cells/ml by the LB medium, and then 2-µl spots (100 cells) were inoculated onto the surfaces of the microtiter plates. The plates were incubated at 30 °C for 24 h. Pseudomonic acid sensitivity was determined as the lowest concentration needed to inhibit cell growth completely.
RESULTS

Pseudomonic Acid Sensitivity of Crude Cell Extract from *P. fluorescens*—How does *P. fluorescens*, producer of the antibiotic pseudomonic acid, avoid suicide? To clarify the precise mechanism of its self-defense, we kinetically analyzed the IleRS activity in crude cell extract from *P. fluorescens* strain NCIB10586. The *Kₘ* for isoleucine was determined to be 10.4 μM, but the *Kₘ* for ATP was observed as biphasic in the Lineweaver-Burk plot and measured as two Michaelis constants (15.2 and 142.9 μM). We could not calculate the *Kᵢ* value for pseudomonic acid precisely from its inhibition curves (Fig. 1A). At 4 mM ATP, 22.7% of the total aminoaacylation activity was drastically inhibited in the presence of as little as 5 μM pseudomonic acid. But the remaining activity could not be abolished; even when the antibiotic was increased to 1 mM, 64.9% of the total activity remained (Fig. 1A). When the activity was measured at 1 mM ATP, 32.1% of the enzyme activity was drastically inhibited in the presence of 100 μM pseudomonic acid, but 45.5% of the activity remained even in the presence of 1 mM pseudomonic acid (Fig. 1B). So we kinetically analyzed the purified IleRS-R1 (the previously reported IleRS) (25) to find that *Kᵢ* values for pseudomonic acid with respect to isoleucine and ATP were calculated as an order of 10⁻⁶ M (Table I). It was curious that the *Kᵢ* values were considerably lower than those measured with crude extract reported so far (14.5 mM) (18). Moreover, the *Kᵢ* values of IleRS-R1 appeared not to be able to explain why *P. fluorescens* is resistant to high concentrations of pseudomonic acid (>1000 μg/ml) (Tables I and II). From these results, we hypothesized that the crude extract from the pseudomonic acid-producing bacteria contains more than one IleRS.

Evidence for Two IleRSs Conferring Distinct Pseudomonic Acid Resistance—This hypothesis was proved by DEAE-Sepharose column chromatography, in which two peaks of IleRS activity were detected (Fig. 2). Western blot analysis using anti-IleRS-R1 antibody showed that the IleRS-R1 was fractionated in the first peak eluting at a lower salt concentration (0.25 M KCl) but not in the second peak (Fig. 3). With the addition of 20 μM pseudomonic acid to IleRS-R1, the peak seen earlier was completely abolished, whereas the second peak (the 0.4 M KCl fraction) was fully active under the same conditions (Fig. 2), suggesting that this fraction contains a newly found IleRS that is highly resistant to pseudomonic acid. Active fractions of pseudomonic acid-resistant IleRS (IleRS-R2) were pooled and purified to homogeneity in five further steps (Fig. 4) as described under “Experimental Procedures.” The *Kᵢ* amino acid sequence of the purified IleRS-R2 protein was determined to be STEGSGPVRFPA. By using SDS-PAGE, the molecular mass of IleRS-R2 was estimated to be 117 kDa, which was slightly higher than that of IleRS-R1 (Fig. 4, lane 6). More surprisingly, IleRS-R2 showed no inhibition in the presence of pseudomonic acid even at the concentration of 5 mM (Table I), 5 orders of magnitude greater than the *Kᵢ* values observed for IleRS-R1 (Table I), and 6 orders of magnitude greater than those for *E. coli* IleRS (2, 25). Aminoaacylation assay solution contains IleRS-R2 enzyme only at a concentration of several nanomolars, indicating that IleRS-R2 has almost no affinity for the antibiotic. So we concluded that *P. fluorescens* possesses two IleRSs, each with a different level of sensitivity to pseudomonic acid. This finding explains why it was difficult to determine *Kᵢ* of the crude extract.

Isolation of New ileS Gene from *P. fluorescens*—To clone the novel ileS gene encoding IleRS-R2, here named ileS2, several degenerate primers were designed from two short sequences highly conserved among eukaryotic IleRSs (17). By using the radiolabeled PCR fragment as a probe, Southern blot hybridization against digested *P. fluorescens* genomic DNA, were cloned and sequenced. In several clones we found a DNA sequence homologous to the ileS gene, which was 52% identical to the corresponding segment of *P. fluorescens* ileS1. To our surprise, it was clear that the peptide predicted from the DNA sequence shared some eukaryote-specific sequences conserved among eukaryotic IleRSs (17). By using the radiolabeled PCR fragment as a probe, Southern blot hybridization against digested *P. fluorescens* DNA and cloning of the full-length ileS2 gene were performed. The probe hybridized to an ~8.5-kb EcoRI fragment by Southern blot analysis (data not shown). On the other hand, the DNA probe derived from ileS1 hybridized specifically to apparently different genomic DNA fragments (data not shown). These results indicate that two distinct genes encode the two IleRSs. An 8.5-kbp fraction of EcoRI-digested *P. fluorescens* DNA was cloned into pTWV228 (Takara). One of the selected candidates obtained by colony hybridization screening was named pR2, and 3093 bp of the structural gene was completely sequenced to identify the entire *P. fluorescens* ileS2. The open reading frame, encoding a protein consisting of 1030 amino acids, was assigned to ileS2, because the deduced amino acid sequence of its 5′-end following a methionine initiator perfectly matched the 12 determined N-terminal residues of IleRS-R2 isolated from *P. fluorescens*. This novel ileS gene encoded a protein with a calculated molecular weight of

![Image](http://www.jbc.org/Downloaded from.../image.png)
Two Isoleucyl-tRNA Synthetases in P. fluorescens

TABLE I

| Strain/plasmid(s) | Pseudomonic acid concentration* | Pseudomonic acid sensitivity (%) |
|-------------------|-------------------------------|---------------------------------|
| NCIB10586         | >1000                         |                                 |
| E. coli/pEXR1 (ileS2) | 800-1000                     |                                 |
| E. coli/pEXR1 (ileS1) | 200                           |                                 |
| E. coli/pEXPCR (E. coli ileS) | 30-40                |                                 |
| E. coli/pEXPCR (vector) | 25-30                  |                                 |

* Pseudomonic acid sensitivities of E. coli DH5α cells transformed with ileS gene-containing plasmid(s) were measured as described under “Experimental Procedures.”

TABLE II

| Strain/plasmid(s) | Pseudomonic acid concentration* |
|-------------------|-------------------------------|
| NCIB10586         | >1000                         |
| E. coli/pEXR1 (ileS2) | 800-1000                   |
| E. coli/pEXR1 (ileS1) | 200                           |
| E. coli/pEXPCR (E. coli ileS) | 30-40                |
| E. coli/pEXPCR (vector) | 25-30                  |

* Pseudomonic acid sensitivities of E. coli DH5α cells transformed with ileS gene-containing plasmid(s) were measured as described under “Experimental Procedures.”

Fig. 2. Elution profile of aminoacylation activity of P. fluorescens IleRS-R1 and IleRS-R2 by DEAE-Sephalcel chromatography. S100 supernatant of P. fluorescens cell extract was applied to a column of DEAE-Sephalcel, and proteins were eluted with a linear gradient of KCl as described under “Experimental Procedures.” IleRS activities in the absence (white circles) or in the presence of 20 μM pseudomonic acid (red circles) are indicated by arrowheads. Protein traces (OD_{280}) are shown as a green line.

Fig. 3. Identification and detection of IleRS-R1 and IleRS-R2 on the fractions of DEAE-Sephalcel by Western blotting with anti-IleRS-R1 (A) and anti-IleRS-R2 (B) antibodies. P. fluorescens IleRS were detected as described under “Experimental Procedures.” The numbers above each lane are the fraction numbers. R1 and R2 indicate IleRS-R1 (50 ng) and IleRS-R2 (50 ng), respectively.

117,738, which was in good agreement with the molecular weight of 117,000 estimated by SDS-PAGE (Fig. 4, lane 6). The G + C content of ileS2 coding sequence (59%) and the codon usage are comparable with those of other conventional P. fluorescens genes.

P. fluorescens IleRS-R2 Is Similar to Eukaryotic IleRSs but Not to Eubacteria—Two consensus sequences of class I aRSs were also found in the P. fluorescens IleRS-R2 sequence: HYGH...
FIG. 5. Sequence alignments of *P. fluorescens* IleRS-R2 with other IleRS. The sequences were aligned by using the ClustalW program (47), and parts of these alignments were optimized and adjusted manually. Eubacterial IleRS that have eukaryote-like features are categorized as type C (light green blocks designated as C beside the sequence alignments), whereas eukaryotic IleRS obtained from eukaryotic cytoplasm are categorized as type E (green blocks designated as E). Conventional eubacterial IleRS are categorized as type B (yellow blocks designated as B), archaebacterial IleRS as type A (red blocks designated as A), and eukaryotic mitochondrial IleRS as type D (orange blocks designated as D). Highly conserved amino acid residues among many IleRS are shown in red, and similar amino acids are shown in orange. Conserved residues among eukaryotic (partially archaebacterial and eubacterial) IleRS are shown in green. Conserved residues among eubacterial and archaebacterial (partially eukaryotic) IleRS are shown in light blue. The KMSKS sequence is highlighted on the bottom line. Dashes represent breaks in actual amino acid sequences of respective proteins to allow sequence alignment (*\textit{Experimental Procedures}*). Numbers at the top correspond to the amino acid residues of *P. fluorescens* IleRS-R2, and those at the bottom correspond to those of *E. coli* IleRS. Each accession number is shown beside the sequence alignment. Psfl2, *P. fluorescens*; Hosac, *Homo sapiens* cytoplasmic; Sacec, *S. cerevisiae* cytoplasmic; Teth, *T. thermophila*; Mytu, *M. tuberculosis*; Stau1, *Staphylococcus aureus*-1; Thth, *T. thermophilus*; Myge, *M. genitalium*; Hosac, *H. sapiens*; Sacec, *S. cerevisiae*; Teth, *T. thermophila*; Mytu, *M. tuberculosis*; Stau1, *S. aureus*-1; Thth, *T. thermophilus*; Myge, *M. genitalium*; Hosac, *H. sapiens*; Sacec, *S. cerevisiae*; Teth, *T. thermophila*; Mytu, *M. tuberculosis*; Stau1, *S. aureus*-1; Basu, *B. subtilis*; Hehy, *Helicobacter pylori*; Hain, *H. influenzae*.

FIG. 6. Complementation Studies—*P. fluorescens* IleRS-R2 was able to charge isoleucine to *E. coli* tRNA^\text{Ile}\text{e*} in *\textit{vitro}* (Fig. 2). To determine whether or not IleRS-R2 can also be functional in *E. coli* cells, the plasmid that carries the *P. fluorescens* IleRS gene was introduced into *E. coli* ileS(ts) mutant Ts331 to see whether it or not would complement IleRS function. These mutant Ts331 cells formed colonies at a non-permissive temperature of 42 °C for 12 h followed by 42 °C for 3 h (Fig. 6, lane 7). On the other hand, IleRS-R1 remained active, and its amount remained unchanged in the soluble fraction of crude extracts prepared after Ts331 cells expressing IleRS-R2 were grown at 30 °C for 12 h followed by 42 °C for 3 h (Fig. 6, lane 7).
Each lane was loaded with 5° Ts331(pEXR2) grown at 30°C and then at 42°C; Ts331(pEXR1) grown at 30°C; lane 5. The natant was then extracted from the following cells: Ts331(pEXPCR) after growth at 42°C from Ts331(pEXR1) cells and from Ts331(pEXR2) cells after the treatment at non-permissive temperatures. S100 supernatants were prepared in the same conditions (Fig. 6).

Resistance to Pseudomonic Acid

To test IleRS-R2-expressing cells' growth in the presence of pseudomonic acid at 40°C/H9262, we preformed experiments with transformants of strain Ts331 at 42°C. We demonstrated that P. fluorescens has two distinct IleRS (IleRS-R1 and IleRS-R2) that are encoded by two distinct ileS genes. E. coli cells containing both ileS genes exerted as much pseudomonic acid resistance as P. fluorescens did. These results explain why P. fluorescens can grow freely in medium containing more than 1000 µg/ml of the antibiotic (Table II). Tables I and II illustrate the fact that, despite a million-fold difference in K values between ileS1 and ileS2, the largest measurable difference in pseudomonic acid sensitivities in vivo is about 5-fold. It is possible that factors other than enzyme sensitivity determine cellular sensitivity. For example, permeation of the antibiotic into the cell membrane may be the cause of the disparity between K values of IleRS enzymes and their cellular resistances in vivo.

IleRS-R1 and IleRS-R2 differed from each other in kinetic, chromatographic, and immunological features as well as in their sensitivity to pseudomonic acid. Why does P. fluorescens maintain two IleRS? Under laboratory conditions, P. fluorescens produces pseudomonic acid at low levels (1–100 µg/ml) during the stationary growth phase and secretes the antibiotic into fermentation broth (1, 33, 34). However, their maximum production of the antibiotic in vivo is not known. The pseudomonic acid-producing bacteria may have required an IleRS that is more tolerant than the relatively sensitive IleRS-R1 to ensure that suicide would not occur in unexpected situations (e.g. in case of over-fermentation) in vivo. In addition, the bacteria may have required a more heat-stable IleRS-R1 to survive, because IleRS-R2 is metabolically unstable at relatively high temperatures. We need to disrupt each ileS gene to clarify their physiological roles in P. fluorescens. Inactivation of ileS2, leading to its killing by pseudomonic acid production, would prove the hypothesis that the IleRS-R2 is there to prevent killing. Two P. fluorescens IleRSs share only 29.6% identical resi-
dues, suggesting that they are evolutionarily distinct from each other. In general, prokaryotes do not have more than one gene encoding for the different aaRSs specific to the same amino acid, although there are a few exceptions for two genes coexisting in the same organism. Two aaRSs encoded by distinct genes are classified into one eubacterial class in which the two show high homology, or they are classified into different eubacterial but not into archaeal/eurkaryotic classes (e.g. E. coli lysS-lysU (35–37), Bacillus subtilis thrS-thrZ (38), Streptomyces coelicolor trpS1-trpS2 (39), and B. subtilis tyrS-tyrZ (40)).

There are also very few cases in which two enzymes are classified into evolutionarily distinct types. One of the two aaRSs belongs to the eubacterial class and the other to the archaeal class (e.g. Thermus thermophilus AspRSs (41), Synecocystis, Bacillus, and Aquifex HisRSs (42)). Besides the chromosomally encoded bacterial IleRS, S. aureus possesses plasmidic eukaryote-like IleRS, which is resistant to pseudomonic acid (21). These cases represent examples of organisms having both eubacterial and archaeal/eurkaryotic types of enzymes. The case of the P. fluorescens IleRS clearly belongs to the latter category, i.e. one prokaryote cell has evolutionarily distinct aaRSs.

Generally, it has been shown that eukaryotic (type E and type C) IleRS are more resistant to pseudomonic acid than are type A and type B IleRS (Table I) (2, 5, 17, 20, 24). Many aaRSs showing eukaryote-like features (type C) have been found recently in several bacteria (12, 42, 43). To date, including S. aureus and P. fluorescens, more than 20 bacteria that possess type C IleRSs have been reported. Although we have not tested the idea thus far, such eukaryotic features strongly suggest that all of these organisms are also resistant to pseudomonic acid. The phylogenetic classification of Mycobacterium tuberculosis IleRS as a eukaryote-like type has been reported, along with the extreme pseudomonic acid resistance of that for IleRS (17). Once the structural basis of the mechanisms for high level pseudomonic acid resistance of that for IleRS (17) is acquired, it appears that particular strains of P. fluorescens possess the eukaryotic ileS gene. This finding supports the gene mobilization hypothesis. If the horizontal transfer of ileS2 is true, when did that event occur? The presence of the S. aureus plasmid carrying a eukaryotic ileS gene provides a candidate for recent horizontal transfer. Unlike the case of S. aureus, the eukaryotic ileS2 gene of P. fluorescens is supposed to be present on its chromosome, according to the results of Southern blotting, plasmid extraction, and cured cell experiments (data not shown). The eukaryotic ileS gene might have been transferred to an ancestor strain of P. fluorescens at some point prior to the recent gene transfer event in S. aureus. The eukaryotic gene has been rearranged and merged into the P. fluorescens chromosome over a long period of evolution. The possibility of horizontal transfers of a pseudomonic acid resistance gene from early eukaryotes to prokaryotes has been proposed (44). That study posited that a eukaryotic ileS gene was first transferred to an unknown bacterium after the divergence of Eukarya and Archaea and was then transferred to several species of bacteria. Although we cannot confirm whether the ancestral P. fluorescens was the first bacterium to acquire the eukaryotic ileS gene, we can easily guess that this producer of pseudomonic acid would have required a pseudomonic acid-insensitive IleRS by the time that antibiotic pseudomonic acid was first produced.

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