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Genetic Analysis of Mutations Causing Borrelidin Resistance by Overproduction of Threonyl-Transfer Ribonucleic Acid Synthetase

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Mutations leading to borrelidin resistance in *Escherichia coli* by overproduction of threonyl-transfer ribonucleic acid synthetase were analyzed genetically. The regulatory mutations were closely linked to the threonyl-transfer ribonucleic acid synthetase structural gene (*thrS*), located clockwise to it. The mutation that causes the threefold-increased enzyme level was more distant from *thrS* than the mutation responsible for the ninefold overproduction. Both mutations were *cis* dominant in merodiploid strains, indicating that they affected promoter-operator-like control elements. Overproduction was restricted to threonyl-transfer ribonucleic acid synthetase and was not observed for the products of genes neighboring *thrS* (e.g., *infC, pheS, pheT*, and *argS*), providing evidence that *thrS* is transcribed singly and that gene amplification is not a likely basis for increased *thrS* expression.

The macrolide antibiotic borrelidin inhibits protein synthesis in procaryotic and eucaryotic organisms by specific interference with the activity of threonyl-tRNA synthetase (10, 22). Some mutations that render cells resistant to this drug affect the primary structure of this enzyme; the altered structure leads either to higher affinity for the natural substrate, L-threonine, or to a preferential decrease of the affinity for the antibiotic (13, 20). Other mutations to resistance increase the intracellular level of wild-type threonyl-tRNA synthetase, augmenting the target molecule of the antibiotic and thereby causing the drug to titrate out within a certain concentration range (13, 20).

The mutations leading to overproduction of threonyl-tRNA synthetase are of particular interest since they provide a means for studying genetic elements involved in the regulation of aminoacyl-tRNA synthetase formation (for review of the regulatory phenomena, see reference 12). The borrelidin resistance mutations are especially valuable for this purpose since they cause the highest genetically stable rise in enzyme activity, up to ninefold, reported in the literature (8, 13). We demonstrate that two of these mutations are linked to the structural gene (*thrS*) of threonyl-tRNA synthetase, that they are situated on the clockwise side of *thrS*, that they are *cis* dominant, and that the gene for threonyl-tRNA synthetase is transcribed independently from closely adjacent cistrons.

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MATERIALS AND METHODS

Strains and media. The genotypes and the derivations of threonyl-tRNA synthetase structural and regulatory mutants of *Escherichia coli* are given in Table 1. Briefly, four mutants were used. GN102 (previously K12B Bor Res 2) and derivatives are borrelidin resistant because of an improved K_m for L-threonine; GN108 (previously K12B Bor Res 8) possesses an enzyme no longer inhibitable by the antibiotic. Both strains are therefore altered in the structural gene for threonyl-tRNA synthetase. GN103 and GN115 (previously K12B Bor Res 3 and K12B Bor Res 15, respectively) possess the wild-type enzyme form but at apparent threefold- and ninefold-increased levels, respectively (8, 13, 20).

The minimal medium used and growth conditions employed were identical to those reported previously (8). Genetic procedures were also the same as published (8, 9). Borrelidin resistance levels were tested on minimal medium plates containing 100 and 120 μg of a crude antibiotic fraction per ml (9). Transductants exhibiting the sensitive phenotype under this condition were checked for their threonyl-tRNA synthetase forms by enzyme assays.

Enzyme assays. In principal, threonyl-tRNA synthetase activity of crude extracts was determined as previously described (20). The following modifications were employed to discriminate between the different mutant enzyme forms. Transductants from crosses between GN108 and the regulatory mutants were tested for their threonyl-tRNA synthetase form in an assay mixture with 20 μM L-[^{14}C]threonine (10 μCi/μmol). Between 1.5 and 4 μg of crude extract protein was used, and in vitro inhibition by borrelidin was determined with 1 μg of antibiotic per ml of assay mixture. In crosses employing structural gene mutant GN102, the L-threonine concentrations in parallel as-

TABLE 1. *Strains of E. coli used*

Strain	Genotype	Source or origin
K10	Hfr (Cavalli) *rel-1 tonA22 T2*^{r}	(1)
GN102	*thrS2*, structural gene mutation	(16)
GN108	*thrS8*, structural gene mutation	(16)
GN103	*thrS3*, regulatory mutation	(16)
GN115	*thrS15*, regulatory mutation	(16)
MC104	*pheS12 pheT354 thi-1 argE3 his-4 proA2 aroD6 lacY1 galK2 mtl-1 xyl-5 tsx-29 supE44*	(3)
MC106	F$^-$ *pheS12 pheT354 pps-4 thi-1 argE3 his-4 proA2 lacY1 mtl-1 xyl-5 tsx-29 supE44*	(3)
KL16-99	Hfr PO45 *thi-1 recA1* λ^-	B. Bachmann, Yale University
MC1699	Hfr PO45 *thi-1 recA1* λ^-	Thermoresistant (42°C) revertant of KL16-99; M. M. Comer, Clark University
GN1031	*thrS3 pps-4 thi-1 argE3 his-4 proA2 lacY1 galK2 mtl-1 xyl-5 tsx-29 supE44*	P1: GN103/MC106
GN1151	*thrS15*, other markers like GN1031	P1: GN115/MC106
GN1081	*thrS8 pps*	EMS[a] mutagenesis
GN1021	*thrS2*, other markers like GN1031	P1: GN102/MC106
GN1032	*thyA* derivative of GN1031	Trimethoprim selection
GN1033	*rpoB recA1* derivative of GN1032	Mating of *rpoB* (Rif^r) derivative of MC1699 with GN1032
GN1152	*thyA* derivative of GN1151	Trimethoprim selection
GN1153	*rpoB, thyA* derivative of GN1152	Mating of *rpoB* (Rif^r) derivative of MC1699 with GN1152
GN1034	Like GN1033, F'148	F' transfer: KLF48/KL159 → GN1033
GN1154	Like GN1153, F'148	F' transfer: KLF48/KL159 → GN1153
KLF48/KL159	*thi-1 his-4 aroD5 proA2 recA1 xyl-5 nalA12 tsx-1*? or *tsx-29* λ^- *supE44*, F' *his*$^+$ *aroD*$^+$ Δ (*cheB-cheC*)	B. Bachmann, Yale University

[a] EMS, Ethyl methane sulfonate.

says were 100 μM and 20 μM, both with and without 1 μg of borrelidin per ml.

Labeling of cultures for two-dimensional electrophoresis. Strains GN102, GN103, GN108, and GN115 were grown in morpholinepropanesulfonate minimal medium (15) at 37°C with 1/10 of the normal K_2SO_4 concentration (0.0276 mM) and supplemented with 0.4% glucose, 40 μM L-isoleucine, 60 μM L-valine, and 80 μM L-leucine (14). Labeling was done for at least six generations in a 5-ml portion of this culture with $^{35}SO_4^{2-}$ (250 μCi/5 ml), and growth was stopped at an absorbance at 420 nm of 1.0. The cells were then mixed with 2.5 ml of a culture of strain K10 grown for at least six doubling times in the same medium (except with 0.276 mM K_2SO_4) in the presence of 40 μCi of L-[^{3}H]leucine per ml. Equilibrium and non-equilibrium two-dimensional gel electrophoresis according to O'Farrell (18, 19) was carried out with the modifications described previously (14). Spot identification after autoradiography was done by coelectrophoresis of 3 μg of purified phenylalanyl-tRNA synthetase, 1 μg of threonyl-tRNA synthetase, or 1 μg of initiation factor 3, which was kindly donated by C. Pon. The positions of arginyl-tRNA synthetase and elongation factor Tu were taken from published information (14). Spots were cut out as described previously (14), soaked in 0.1 ml of water at 4°C overnight, and digested for 24 h with a 1:10 mixture of Lumasolve and Lipoluma (LKB Instruments). ^{3}H counts were corrected for overlapping ^{35}S radioactivity, and the ^{35}S/^{3}H counts in each spot were divided by the ^{35}S/^{3}H counts for the total protein of the respective strain. The ^{35}S/^{3}H ratio for total protein was determined by precipitation of the protein of 10 μl of the doubly labeled extract with 10% trichloroacetic acid, filtering and washing on GF/C glass-fiber filters, and counting in the same scintillation fluid as mentioned above. The ^{35}S/^{3}H ratio for the threonyl-tRNA synthetase of strain GN102 was determined by cutting out equal areas from the positions of the structurally altered enzyme (given by the autoradiogram) and the wild-type enzyme (given by the purified wild-type marker protein).

RESULTS

Genetic location of threonyl-tRNA synthetase regulatory mutations. Previous genetic analysis has shown that *thrS* (the structural gene for threonyl-tRNA synthetase) is closely linked to the *pheS pheT* cistrons (genes for the α and β subunits of phenylalanyl-tRNA synthetase [5]) (9, 23). To determine the location of the mutations causing threonyl-tRNA synthetase overproduction the crosses listed in Table 2 were performed. Besides a check for the borrelidin resistance phenotype on plates, enzyme assays were carried out on 100 transductants from the first cross and 50 transductants from the second cross. The results indicated that the regulatory mutation in threonyl-tRNA synthetase-overproducing strains GN115 and GN103

TABLE 2. *Transductional mapping of mutations leading to threonyl-tRNA synthetase overproduction*[a]

Donor	Markers			Frequency (%) of transductant classes
GN115	*pheT*$^+$	*pheS*$^+$	*thrS15*	54
(*thrS15*)	*pheT354*	*pheS12*	*thrS*$^+$	41
	pheT$^+$	*pheS12*	*thrS*$^+$	2
	pheT$^+$	*pheS*$^+$	*thrS*$^+$	3
GN103	*pheT*$^+$	*pheS*$^+$	*thrS3*	50
(*thrS3*)	*pheT354*	*pheS12*	*thrS*$^+$	44
	pheT$^+$	*pheS12*	*thrS*$^+$	6

[a] Recipient: MC104 (*pheS12 pheT354 thi-1 argE3 his-4 proA2 aroD6 lacY1 galK2 mtl-1 xyl-5 tsx-29 supE44*). The selected marker was *aroD*$^+$; 100 recombinants of each class were tested for *p*-fluorophenylalanine resistance (*pheS12*), for temperature sensitivity (*pheT354*), and for borrelidin resistance (*thrS*). The specific activities of threonyl-tRNA synthetase of 100 transductants from the first cross and 50 transductants from the second cross were determined by enzyme assay. Recombinant classes other than those listed were not obtained.

was cotransducible with *aroD* and, similar to *thrS* structural gene mutations (9), was closely linked to the *pheS pheT* cistrons. The gene order delineated from the distribution of recombinant classes (Table 2) was *aroD-pheT-pheS-thrS*. In each case, the overproduction was transduced fully.

The regulatory mutation is *cis* dominant. To determine whether the regulatory mutation was *cis* or *trans* dominant, episome F'148 was transferred into *recA* derivatives of the overproducing strains. (For their construction, see Table 1.) F'148 carries the *thrS* gene, the presence of which leads to an increase in threonyl-tRNA synthetase synthesis (21).

The episome was maintained by selection for Pps$^+$ and His$^+$ phenotypes, i.e., for the presence of genes which flank *thrS* on both sides. The specific threonyl-tRNA synthetase of merodiploids was examined to determine whether the presence of a wild-type *thrS* regulatory element on the episome influenced the increased expression of the chromosomal *thrS* gene. Table 3 shows the results for extracts from cells grown in minimal medium. The presence of F'148 in the cells did not repress the increased level of the chromosomal gene product. Rather, a small, reproducible increase was observed, which is in accordance with the observation that aminoacyl-tRNA synthetase formation is largely gene dose dependent (3, 7, 9, 21). Thus, the regulatory element involved in increased threonyl-tRNA synthetase expression is *cis* dominant and, therefore, may affect promoter-operator-like control regions.

Genetic mapping of the regulatory mutation relative to the *thrS* structural gene. The precise location of the regulatory mutation relative to *thrS* mutations was established by crossing structural gene mutants with regulatory gene mutants. Both lead to a borrelidin resistance phenotype, and therefore the number of wild-type (borrelidin-sensitive) recombinants in reciprocal crosses allows the delineation of the order of mutations (Fig. 1) under the assumption that the relative frequency of wild-type, i.e., antibiotic-sensitive, recombinants is a measure of whether double or quadruple crossover events are necessary for their formation.

Table 4 gives the result of such crosses between two regulatory and two structural gene mutants. Transductants with wild-type threonyl-tRNA synthetase were first detected by their growth response to borrelidin, and their identification was confirmed by enzyme assay. In transductions 1 and 2, the *thrS8* structural gene mutant was used as the donor, and the regulatory gene mutants were used as recipients. The frequency of borrelidin-sensitive recombinants obtained was lower than that for the reciprocal crosses (transductions 3 and 4). This indicates that fewer crossover events were necessary to obtain the wild-type phenotype when the structural gene mutation was present in the recipient (transductions 3 and 4). Since the quantitative difference, however, was small, we used a second *thrS* structural gene mutation

TABLE 3. *Specific activities of threonyl-tRNA synthetase in ribosome-free crude extracts of merodiploid strains*

Strain	Threonyl-tRNA synthetase activity (nmol/mg of protein)
MC106 *thrS*$^+$	5.1
GN1033 *thrS3*	20.5
GN1034 *thrS3*/F'*thrS*$^+$	21.8
GN1153 *thrS15*	34.0
GN1154 *thrS15*/F' *thrS*$^+$	37.7

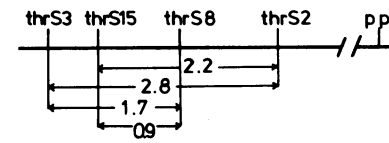

FIG. 1. *Mapping of the thrS regulatory gene mutations relative to structural gene mutations. Numbers refer to frequency of crossover events between markers (see Table 4).*

TABLE 4. *Transductional ordering of regulatory mutations relative to thrS structural gene mutations*

Cross	Donor	Recipient[a]	Total no. tested	Transductants with wild-type form and amount of threonyl-tRNA synthetase (%)
1	GN108 (*thrS8*)	GN1151 ((*thrS15 pps*)	2,000	0.5
2	GN108 (*thrS8*)	GN1031 (*thrS3 pps*)	2,000	1.0
3	GN115 (*thrS15*)	GN1081 (*thrS8 pps*)	2,000	0.9
4	GN103 (*thrS3*)	GN1081 (*thrS8 pps*)	2,000	1.7
5	GN102 (*thrS2*)	GN1151 (*thrS15 pps*)	500	0.8
6	GN102 (*thrS2*)	GN1031 (*thrS3 pps*)	500	1.8
7	GN115 (*thrS15*)	GN1021 (*thrS2 pps*)	500	2.2
8	GN103 (*thrS3*)	GN1021 (*thrS2 pps*)	500	2.8

[a] Selection was in each case for Pps$^+$.

(*thrS2*) to substantiate this result. The data of crosses 5 and 6 and of reciprocal transductions 7 and 8 indeed confirmed the results. The order of mutations given in Fig. 1 is, therefore, the most plausible one since it results in a wild-type genotype by a double crossover with the regulatory mutants as donors, whereas a quadruple event is needed in case of the *thrS* structural gene mutant. The *thrS3* site also seems farther away from each of the two structural gene mutations than does the *thrS15* site.

Gel electrophoretic analysis of *thrS* mutants. Analysis of the *thrS* mutants on O'Farrell gels (18, 19) was performed (i) to possibly provide direct evidence for a structural alteration of threonyl-tRNA synthetase in strains GN102 and GN108, (ii) to demonstrate that the enzyme in the overproducing strains consists of the wild-type form, and (iii) to investigate whether products from genes which are immediately adjacent to *thrS* are also overproduced, since if they are present in the normal amount, this would strongly argue against gene amplification as the cause for overproduction. The latter information was necessary since crosses 3 and 4 of Table 4 (but none of the other crosses) generated transductants which, when belonging to the overproducing class, were not fully parental in their threonyl-tRNA synthetase level but showed only about 50% of the specific enzyme activity (wild-type form) of the initial regulatory mutant. This could be due to the existence of an additional mutation in strain GN108 which reduces the full expression of the regulatory mutation. Figure 2 shows relevant sections from two-dimensional gels. Strain GN102 clearly possessed an altered threonyl-tRNA synthetase (Fig. 2A); the enzyme of GN108 seemed to migrate in the same position as the wild-type enzyme or only slightly different from it (Fig. 2B). Parts C and D of Fig. 2 show that the threonyl-tRNA synthetase of the overproducing strains was wild-type by electrophoretic criteria.

The relative amounts of threonyl-tRNA synthetase and of other proteins, including those coded for by genes in the immediate neighborhood of *thrS*, were assessed by measuring the radioactivity in protein spots from gels of doubly labeled extracts (Table 5). The genes of initiation factor 3 (*infC*) and the α and β subunits of phenylalanyl-tRNA synthetase (*pheS* and *pheT*) are directly adjacent to *thrS* on the counterclockwise side; that for arginyl-tRNA synthetase (*argS*) is about 1 min away on the clockwise side. The products of these genes were present in equal amounts in all four strains. Threonyl-tRNA synthetase itself, in contrast, was present in greatly varying amounts. Relative to the enzyme level of GN108, a more than twofold- and fivefold-increased enzyme level was determined for GN103 and GN115, respectively. Since previous measurements by other techniques resulted in three- and ninefold overproduction values (8, 13) relative to that of the wild-type, this indicates that GN108 itself might have an increased threonyl-tRNA synthetase level. As indicated by the relative intensity of the threonyl-tRNA synthetase spot in Fig. 2A, strain GN102 possesses an increased amount of the structurally altered polypeptide.

Since GN102 was isolated as a single-step, spontaneous borrelidin-resistant mutant, one can assume that the structural alteration causes overproduction. This assumption is also supported by the finding that GN102 can be transduced to full borrelidin sensitivity when the *thrS2* site is replaced (Table 4).

DISCUSSION

The mode of regulation of the intracellular level of aminoacyl-tRNA synthetases is still not fully understood, although it has been proven that the rate of formation of these enzymes is affected on one hand by special growth conditions and on the other hand by genetic control; both factors affect the concentration of amino-

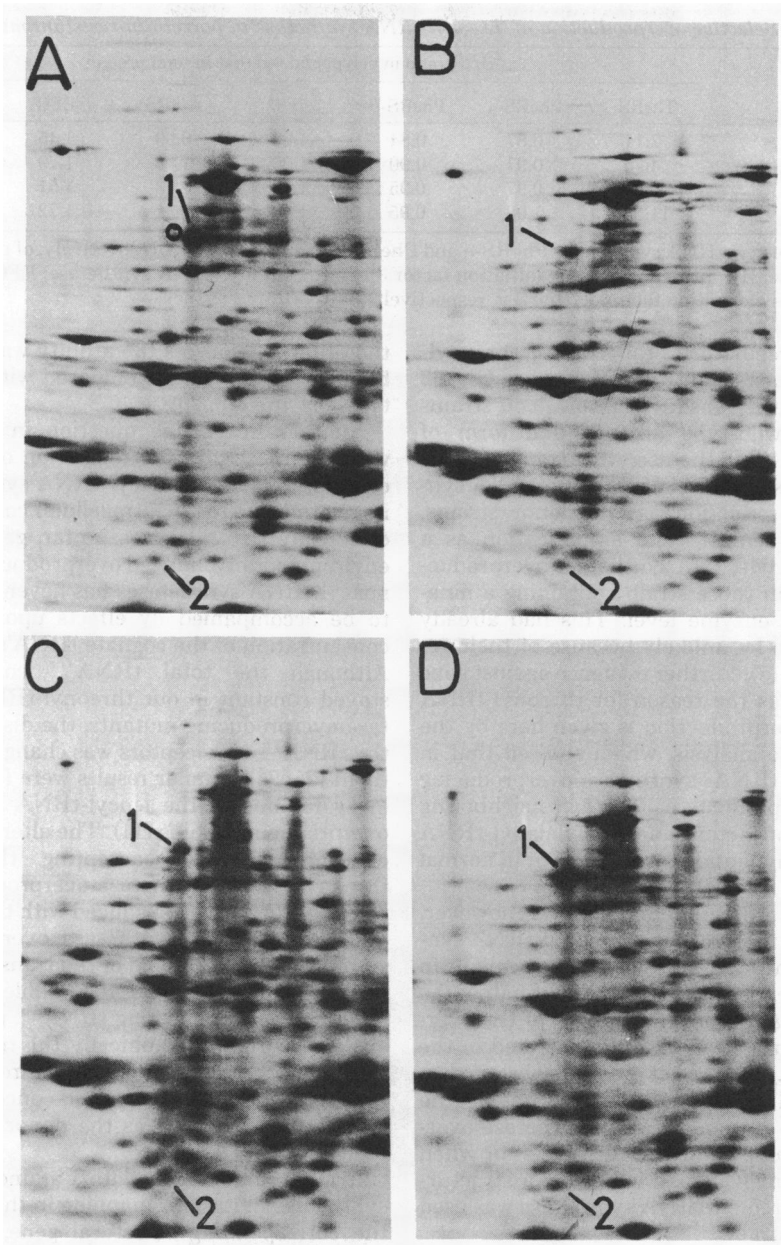

FIG. 2. *Sections from two-dimensional electropherograms (13) of proteins from (A) strain GN102, (B) strain GN108, (C) strain GN103, and (D) strain GN115. The positions of threonyl-tRNA synthetase and the α subunit of phenylalanyl-tRNA synthetase are indicated by (1) and (2), respectively. The circle in (A) indicates the migration position of purified wild-type threonyl-tRNA synthetase.*

acyl-tRNA synthetases individually, or sometimes certain groups of aminoacyl-tRNA synthetases are varied simultaneously (12, 16, 17).

Here we showed that mutations leading to overproduction of wild-type threonyl-tRNA synthetase map outside but in the vicinity of the *thrS* gene. This result is similar to the one reported for *E. coli* mutants that overproduce structurally altered leucyl- or valyl-tRNA synthetases (2, 11). In those mutants, as in our threonyl-tRNA synthetase-overproducing strains, the mutation responsible for the aminoacyl-tRNA synthetase overproduction is *cis* dominant, thus in either case pointing toward a mutation in a promoter-operator-like regulatory element.

TABLE 5. *Selective overproduction of threonyl-tRNA synthetase in borrelidin-resistant mutants*[a]

Strain	$^{35}S/^{3}H$ ratio in polypeptide to that in total protein						
	ThrRS	PheRS-α	PheRS-β	IF3	ArgRS	EFG	EFTu
GN102	7.11	0.87	0.84	1.03	0.99	1.45	1.05
GN103	4.75	0.97	0.90	1.05	1.16	1.79	1.21
GN108	2.21	0.97	0.95	1.18	1.13	1.51	1.38
GN115	11.35	1.08	0.95	1.10	1.31	1.72	1.18

[a] ThrRS, Threonyl-tRNA synthetase; PheRS-α and PheRS-β, α and β subunits, respectively, of phenylalanyl-tRNA synthetase; IF3, protein synthesis initiation factor 3; ArgRS, arginyl-tRNA synthetase; EFG and EFTu, protein synthesis elongation factors G and Tu, respectively.

When the regulatory properties of threonyl-tRNA synthetase-overproducing strains GN103 and GN115 were transduced into *E. coli* strains bearing the wild-type amount and form of threonyl-tRNA synthetase, the transductants exhibited the same level of threonyl-tRNA synthetase overproduction as the donor strains, thus arguing against gene amplification as a cause of threonyl-tRNA synthetase overproduction, at least in those strain containing a ninefold-increased enzyme level. This had already been shown to be unlikely because of their genetic stability (8). Further evidence against gene amplification as the reason for threonyl-tRNA synthetase overproduction is given here by the electrophoretic analysis, which showed that in the threonyl-tRNA synthetase-overproducing mutants, the products of the *thrS* neighboring genes, initiation factor 3 and phenylalanyl-tRNA synthetase polypeptides, were present in normal amounts.

A result not fully understood so far, however, is that when structural gene threonyl-tRNA synthetase mutant GN108 is used as a recipient in transduction experiments, only about half of the threonyl-tRNA synthetase activity of the overproducing donor strains can be detected in the transductants. This effect was not observed in the reciprocal crosses (with GN108 being the donor and the threonyl-tRNA synthetase-overproducing strains being the recipients) or when another mutant (GN102) exhibiting a structurally altered threonyl-tRNA synthetase was used as the donor strain.

From these data we deduce that mutant GN108, besides the mutation in the *thrS* gene, possesses another genetic lesion which affects threonyl-tRNA synthetase activity directly or indirectly; this effect is not observed when mixing crude extracts of wild-type strains and this mutant in vitro (13). This property of GN108 correlates with an other peculiar feature: from all 12 borrelidin-resistant mutants possessing a structurally altered threonyl-tRNA synthetase, only the enzyme of GN108 showed a lowered affinity for its substrate, threonine, whereas in the other 11 mutants the affinity was increased by a factor of 5 to 20 compared with the wild-type enzyme (13, 20).

Another interesting question in connection with the mechanism of regulation of the intracellular level of aminoacyl-tRNA synthetases is its relationship to the intracellular concentration of cognate tRNA species: so far, genetically or environmentally induced overproduction of aminoacyl-tRNA synthetases has never been found to be accompanied by effects upon the total concentration of the cognate tRNA's (4, 11, 25). Although the total $tRNA^{Thr}$ concentration stayed constant in our threonyl-tRNA synthetase-overproducing mutants, the distribution of the $tRNA^{Thr}$ isoacceptors was changed in one of them (25, 26). Similar results were found previously for one of the leucyl-tRNA synthetase-overproducing strains (11). The alteration of the concentration of isoaccepting $tRNA^{Thr}$ in threonyl-tRNA synthetase-overproducing mutant GN103 was cotransduced with the property of threonyl-tRNA synthetase overproduction, since when tRNA of one of the transductants of the cross GNA103 × MC104 (Table 2) was isolated and its isoaccepting $tRNA^{Thr}$ pattern was analyzed chromotographically, this transductant exhibited the same increased threonyl-tRNA synthetase activity and altered concentration of isoaccepting $tRNA^{Thr}$ as the donor strain (unpublished data).

In addition to the regulatory aminoacyl-tRNA synthetase mutations mapping in the vicinity of the corresponding structural gene, there is a second class which maps at distant loci on the *E. coli* chromosome, as reported for the leucyl- and glutaminyl-tRNA synthetases (4, 11, 24). With the exception of one of the leucyl-tRNA synthetase mutants, the overproduction of the individual aminoacyl-tRNA synthetases is restricted to the mutant form of the enzyme, since when that lesion is present in wild-type strains, the wild-type aminoacyl-tRNA synthetase concentration is not affected (4, 11, 24).

For the regulatory mutations mapping far from the structural gene, no altered pattern of

cognate isoaccepting tRNA's has been reported. However, in this connection, it is worth mentioning a mutant which is unable to derepress its isoleucyl-tRNA synthetase in response to isoleucine deficiency and which exhibits an altered pattern of isoleucine accepting tRNA's; the underlying mutation maps neither in the *ileS* gene nor in any of the known genes coding for $tRNA^{Ile}$ (6).

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