CURRENT MICROBIOLOGY Vol. 25 (1992), pp. 257–260

Current Microbiology

Role of Nalidixic Acid in Isolation of *Salmonella typhimurium* Strains Capable of Growth at 48°C

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Abstract. *Salmonella typhimurium* thermotolerant mutants dependent on the presence of nalidixic acid for growth at 48°C were isolated and designated nalidixic acid-dependent, thermotolerant mutants, *nal*^{d}*ttl*. Genetic mapping revealed that *nal*^{d}*ttl* alleles map within the *gyrA* gene. When *S. typhimurium* strain Q was plated in the dark on nutrient agar containing nalidixic acid (20 μg/ml) as a photosensitizer and briefly exposed to white light or near VU light prior to incubation at 42°C, nalidixic acid-resistant mutants arose in about 16 h at frequencies of 5×10^{-8} for white light and 1×10^{-6} for near UV light. About 10% of these nalidixic acid-resistant mutants derived from photodynamic mutagenesis exhibited the thermotolerant characteristic.

Previously we isolated thermophilic derivatives of *Bacillus subtilis* and *Bacillus pumilus* [1]. Two mutations are responsible for growth at the thermophilic temperatures (up to 70°C). These thermophilic *B. subtilis* mutants were found to be nalidixic acid resistant. Furthermore, nalidixic acid-resistant *B. subtilis* mutants have been isolated that are able to grow about 6°C above the upper temperature limit for growth (50°C) of the parental strains (M.L. Droffner and N. Yamamoto, unpublished observations). These results suggest that a mutation within the gyrase gene is one of the two mutations required for thermophilic growth of this species. We have isolated thermotolerant mutants capable of growth at 48°C of *Escherichia coli, Salmonella typhimurium,* and *Pseudomonas aeruginosa* [2–4]. All of these thermotolerant mutations map within the *gyrA* locus. These observations suggest that mutation from mesophile to thermotolerant is feasible in a variety of bacterial genera, and these thermotolerant mutants might be easily isolated by selecting on nalidixic acid-supplemented nutrient agar plates.

In the present communication, we report isolation procedures for thermotolerant mutants of *S. typhimurium* involving the use of nalidixic acid. These mutants can be dependent on the presence of nalidixic acid for growth at 48°C. Photodynamic mutagenesis by nalidixic acid and light or a prolonged incubation with nalidixic acid in the dark can be used for isolation of nalidixic acid-resistant thermotolerant mutants and nalidixic acid-dependent thermotolerant mutants.

Materials and Methods

Bacteria and phage. Three genetically well-characterized *S. typhimurium* strains (Q, LT-2, and LT-7) were used in these studies. These mesophiles are able to grow at temperatures up to 42°C. Phage P22 was used for cotransduction analyses.

Media. Media used were described elsewhere [2]. Nalidixic acid, tetracycline, and rifampicin were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Anaerobic growth conditions. The anaerobic gas mixture and the procedure for anaerobic growth conditions were described previously [10].

Isolation of thermotolerant mutants. Nalidixic acid-dependent, thermotolerant mutants able to grow at 48°C were isolated by plating a freshly grown culture of *S. typhimurium* Q on nutrient agar plates containing 20 μg nalidixic acid/ml and were incubated for 4 days at 48°C. Nalidixic acid-resistant mutants of *S. typhimurium* Q carrying the thermotolerant phenotype were also isolated by plating on nutrient agar plates containing 20 μg nalidixic acid/ml and incubating at 42°C for about 4 days. For prolonged incubation, nutrient agar plates were placed in candle jars to prevent extensive dehydration of agar. For photodynamic mutagenesis of *S. typhimurium,* strain Q was plated on nutrient agar containing nalidixic acid (20 μg/ml) as a photosensitizer, exposed to near UV light (Black-light-Blue, Sylvania F9T5/BLB adjusted at a fluence of 1 Wm^{-2}) or white light (white fluorescent, Sylvania F9T5 adjusted at a fluence of 2 Wm^{-2}) for 8 and 16 s respectively

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Table 1. Growth of *Salmonella typhimurium* and its gyrase mutants with or without nalidixic acid at various temperatures

Strain	Concentration of nalidixic acid in nutrient agar (μg/ml)	Temperature						Reference
		30°C	37°C Aer	37°C Ana	42°C	48°C	50°C	
Q (parental)	20	−	−	−	−	−	−	Boyd strain
	0	+	+	+	+	−	−	
Q*nal*^{d}*ttl*	20	+	+	+	+	+	−	This paper
	0	+	+	+	+	−	−	
Q*nal*^{di}*ttl*	20	+	+	+	+	+	−	This paper
	0	+	+	+	+	+	−	
Q*nal*^{r}*ttl*	20	+	+	+	+	+	−	[2, 3, 5]
	0	+	+	+	+	+	−	
Q*nal*^{r}	20	+	+	+	+	−	−	[2]
	0	+	+	+	+	−	−	
Q*nal*^{r}Aer	20	+	+	−	+	−	−	[12]
	0	+	+	−	+	−	−	

Abbreviations: +, growth; −, no growth; Aer, aerobic growth condition; Ana, anaerobic growth condition; *nal*^{d}, nalidixic acid dependent; *nal*^{di}, nalidixic acid independent; *ttl*, thermotolerant; *nal*^{r}*Aer*, nalidixic acid resistant/strict aerobic mutant.
Note: Nalidixic acid is thermostable for many days under these conditions.

and incubated at 42°C for 16 h to detect nalidixic acid-resistant colonies.

Mapping. *S. typhimurium* LT-2 carrying transposon Tn10, *zeh-597*::Tn10, inserted at 45 min and 95% linked to *gyrA* [8], was used to determine the P22 cotransduction frequency of Tn10 and *gyrA*.

Results and Discussion

S. typhimurium Q, LT-2, and LT-7 grow well at mesophilic temperatures with generation times of about 30 min at 37°C, but did not grow above 42°C. When overnight (37°C) cultures of *S. typhimurium* Q were spread on nutrient agar plates containing 20 μg nalidixic acid/ml and incubated at 48°C for about 4 days, thermotolerant mutants capable of growth at 48°C only in the presence of nalidixic acid were isolated (Table 1). The generation time of their growth at 48°C was about 30–40 min. These mutants were unable to grow at this elevated temperature in the absence of nalidixic acid. At 37°C, however, they grew with or without the presence of nalidixic acid. These mutants were designated as nalidixic acid-dependent, thermotolerant mutants (*nal*^{d}*ttl*) because nalidixic acid is required for growth at 48°C but not for growth at mesophilic temperatures. Serial cultivations of *nal*^{d}*ttl* mutants on nalidixic acid-supplemented nutrient agar at 48°C resulted in overgrowth of nalidixic acid-independent, thermotolerant (*nal*^{di}*ttl*) mutants, which do not require nalidixic acid for growth at the elevated temperature but retain nalidixic acid resistance.

Unlike the nalidixic acid-resistant strict aerobic mutants (*nal*^{r}*Aer*) [10, 11] which lack DNA gyrase activity and thus are nalidixic acid resistant, the *nal*^{d}*ttl* mutants were able to grow anaerobically at 37°C, as shown in Table 1. Presumably the DNA gyrase is active under these conditions because increased negative superhelicity is required for anaerobic growth [10, 11]. Since the target of nalidixic acid is gyrase A subunit [9] and *nal*^{d}*ttl* mutants require nalidixic acid for thermotolerant growth at elevated temperature, the *nal*^{d}*ttl* mutation appears in the absence of nalidixic acid to produce a dysfunctional gyrase A subunit at 48°C. This dysfunctional subunit is amendable by complexing with nalidixic acid to produce an active form of the enzyme. Therefore, a mutation within the *gyrA* locus must have occurred, and the nalidixic acid dependency at 48°C strongly suggests that a specific alteration of the gyrase activity, thus a certain superhelicity, is required for thermotolerance. It seems likely that *gyrA* mutation gives rise to altered DNA superhelicity, causing pleiotropic effects on the various genes [10] and thus changing the expression of genes necessary for growth at elevated temperatures. From these findings, it is unlikely that *nal*^{d}*ttl* mutants can be isolated from gyrase A subunit defective or deletion strains. In fact, five attempts to isolate *nal*^{d}*ttl* thermotolerant mutants from the *E. coli* C600 *gyrA*::Mu-*lac* fusion strains [11], which are presumably devoid of gyrase molecules, were unsuccessful.

The *nal*^{d}*ttl* mutation in *S. typhimurium* Q was mapped as with our previously described nalidixic

Table 2. Mapping the *S. typhimurium* *nal*^{d}*ttl* alleles by cotransduction of a Tn10 transposon with P22 phage

Exp.	Donor	Recipient	No. of selected markers	No. of unselected markers	Percent cotransduction
Ia	*zeh-597*:Tn10	Q*nal*^{d}*ttl*	158 *tet*^{r}	149 *nal*^{S}, 9 *nal*^{d}*ttl*[a]	94.3%
Ib	Q*nal*^{d}*ttl*	*zeh-597*::Tn10	89 *nal*^{d}*ttl*	82 *tet*^{S}, 7 *tet*^{r}	92.1%
II	Q*nal*^{d}*ttl*	LT-2*leu500*	48 *nal*^{r}[b]	48 *nal*^{d}*ttl*	100%
	Q*nal*^{d}*ttl*	Q*rif*^{r}	25 *nal*^{r}	25 *nal*^{d}*ttl*	100%
	Q*nal*^{d}*ttl*	LT-7*proAB*	21 *nal*^{r}	21 *nal*^{d}*ttl*	100%

Ia and Ib, mapping of *nal*^{d}*ttl* by P22 cotransduction of a Tn10 transposon; II, transduction of the *nal*^{d}*ttl* allele into other *S. typhimurium* strains by P22 phage.

Abbreviations: *ttl*, thermotolerant mutants; *nal*^{d}, nalidixic acid-dependent growth; *nal*^{r}, resistant to 20 μg/ml nalidixic acid; *nal*^{s}, nalidixic acid sensitive; *tet*^{r}, tetracycline resistant, 10 μg/ml; *tet*^{S}, tetracycline sensitive; *rif*^{r}, rifampicin resistant.

[a] Observed at 48°C on nutrient agar, 20 μg nalidixic acid/ml.

[b] Selected as *nal*^{r} at 37°C on nutrient agar containing 20 μg nalidixic acid/ml and tested for *nal*^{d}*ttl* as dependent on nalidixic acid for growth at 48°C.

acid-resistant, thermotolerant (*nal*^{r}*ttl*) mutants [2, 5] which are resistant to but do not require nalidixic acid for growth at the elevated temperature [2, 3, 5]. After a *nal*^{d}*ttl* mutant was infected with P22 phage previously grown on a transposon *zeh-597*::Tn10 carrier, tetracycline-resistant transductants were selected at 37°C, and the number of cotransduced wild-type *gyrA*$^{+}$ marker, as determined by nalidixic acid sensitivity (*nal*^{s}), was scored. As shown in Table 2, Exp. Ia, the *nal*^{d}*ttl* mutation was 94.3% linked to the Tn10 transposon. In addition, the *zeh-597*::Tn10 carrier was infected with P22 previously grown on Q*nal*^{d}*ttl* and selected for nalidixic acid resistance. As shown in Table 2, Exp. Ib, *nal*^{d}*ttl* is 92.1% cotransduced with tetracycline sensitivity. Thus, it is concluded that the *nal*^{d}*ttl* allele is located within the *gyrA* locus. Moreover, this *nal*^{d}*ttl* mutation allele was easily transduced to an isogenic rifampicin-resistant mutant Q*rif* and auxotrophic strains LT-2 *leu*500 and LT-7 *pro*AB and selected as nalidixic acid resistance at 37°C. All nalidixic acid-resistant transductants were found to be thermotolerant (Table 2, Exp. II).

Nalidixic acid-resistant mutants were also isolated by spreading a clone of *S. typhimurium* Q on nutrient agar plates containing 20 μg nalidixic acid/ml and exposing to white light for 16 s or black light for 8 s, followed by 16 h incubation at 42°C. The frequencies of these mutations were 5×10^{-8} for white light and 1×10^{-6} for near UV light. Nalidixic acid is a near UV photosensitizer capable of production of singlet oxygen [7]. Light illumination to bacterial cells in the presence of a singlet oxygen producer causes photodynamic mutation [6]. About 10% of the nalidixic acid-resistant mutants isolated by photodynamic conditions were thermotolerant. Like chemical and UV mutagenesis, photodynamic mutations appear to occur at random nalidixic acid-resistant mutation sites. Specific sites (10%) of nalidixic acid-resistant mutation alleles produce a certain superhelicity required for growth under the thermotolerant environment. This is in agreement with our previous observation that about 10% of nalidixic acid-resistant mutants derived from chemical mutagenesis are also thermotolerant [5; M.L. Droffner and N. Yamamoto, unpublished observations]. Moreover, photosensitizing mutagenic activity of nalidixic acid might explain the observation that nalidixic acid-dependent mutants eventually became nalidixic acid-independent, resistant mutants after serial cultivations in the presence of nalidixic acid with inadvertently repeated exposure to ambient light. Awareness of these problems is essential for genetic analysis and maintenance of mutants in the presence of nalidixic acid because genetic stability of mutants and transductants depends on avoiding reversion and suppressor mutations by photodynamic mutagenesis.

Although cultivation with nalidixic acid in the dark prevented photodynamic mutation, a prolonged (4-day) cultivation on nalidixic acid containing agar plates at 42°C appeared to select thermotolerant mutants. *E. coli* and *P. aeruginosa* were also found to produce thermotolerant mutants by the above procedure [M.L. Droffner and N. Yamamoto, unpublished observations]. These mutants of three different genera, along with nalidixic acid dependency of thermotolerant *S. typhimurium* mutants, showed that nalidixic acid plays an important role in mutagenesis and selection of mutants able to grow at elevated temperatures.

ACKNOWLEDGMENT

This investigation was supported in part by National Science Foundation grant no. DMB-8511651 to N.Y.

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