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Isolation and Characterization of Nalidixic Acid Resistant Mutant of a Thermophilic Actinomycete

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Abstract: The bactericidal action of nalidixic acid on the wild type strain 1227 of an obligate thermophile *Thermoactinomyces vulgaris* Tsilinsky has been studied. A new, hitherto unreported, nalidixic acid resistant strain (nal^r) isolated, has exhibited auxotrophy for asparagines and lysine, with an increased resistance to UV rays as well as hyperosmotic shock, with maximum bactericidal concentration (MBC) 200µg ml^{-1}.

Key words: Nalidixic acid · Gyrase inhibitor · *Thermoactinomyces* · Mutant

INTRODUCTION

Nalidixic acid and other quinolones are potent bactericidal agents that inhibit DNA synthesis by trapping gyrase and topoisomerase IV, as ternary complexes causing single / double strand DNA breaks [1, 2]. The mechanism(s) of action of nalidixic acid have been investigated in gram +ve and gram -ve bacteria e.g. in *E. coli* [3, 4], in *Bacillius* and in *Psudomonas aeruginosa* [5]. A number of cascade events on the cell due to action of nalidixic acid have been reported [6, 7] such as blockage of DNA and RNA synthesis, SOS response, change in cell permeability and proliferation leading to caseation of cells.

T. vulgaris is an obligate thermophilic actinomycete that grows optimally at 52°C. It produces thick walled endospores generally refractile to a large number of mutagens. These endospores can also withstand extreme natural stress and remain viable for long years [8]. Rifampicin resistant mutant has been isolated earlier in this bacterium, which invariably exhibits auxotrophy for isoleucine and lysine [9].

The current study deals with the action of the nalidixic acid on *T. vulgaris*, the isolation of a new nalidixic acid resistant strain (nal^r) and its preliminary characterization.

MATERIALS AND METHODS

A clonal culture of wild type strain 1227 of *T. vulgaris*, obtained from the John Innes Institute, Norwich, England was maintained in laboratory on minimal medium with casein (MMC) pH 6.6 - 6.8 as prescribed [10] with slight modification ($NaNO_3$ - 2.0 gs, KCl - 0.5 g, $MgSO_4.7H_2O$ - 0.5 g, â Sodium glycerophosphate - 0.5g, $FeSO_4$ - 0.1 g, Sucrose - 30 gs and Casein - 6.0 gs $litre^{-1}$). Agar (1.5% w/v) was added as and when required.

MMS (Minimal medium synthetic) was used to test for auxotrophy for amino acids. It was prepared by adding six amino acids namely L- alanine, L- arginine, L- asparatic acid, L- methionine, L- threonine and L- valine (1mM each), with 0.0015 % (w/v) spermidine phosphate to the ingredients of minimal medium without casein [10]. Nalidixic acid (Sigma, USA) dissolved in 0.5 (N) NaOH and was filtered and sterilized before use.

Selection of Nalidixic Acid Resistant Mutant (nal^r): The wild type strain 1227 of *T. vulgaris* was grown on solid MMC plates at 52°C. The spores were collected in sterile normal saline after 24h of incubation by scrapping with a sterile wire loop. The spore suspension (10^6) was vortexed in a mixer. The spore suspension was further diluted and 0.1 ml of the diluted solution spread over MMC plates containing different concentrations of nalidixic acid ranging from 50µg ml^{-1} to 250µg ml^{-1}.

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The colonies appearing after incubation period ranging from 24h to 96h in a plate were transferred, by replica plating method, to another plate containing next higher concentration of the drug. The surviving colonies isolated from MMC plates containing 150µg ml^{-1} of nalidixic acid were scored as nal^r mutant. The selected nal^r mutants were kept in Master plate. Growths of wild and mutant strains after 8h in shake culture were recorded in terms of absorbance (OD) at 480nm.

Test for Auxotrophy: Plating of the mutant colonies, on a MMS media containing different amino acids, was done to test for the auxotrophy. Wild type 1227 was used as the control.

UV Irradiation: Cultures (10^6 spores) were grown in conical flask on MMC liquid medium on a gyratory shaker at 150 rpm for 4h up to mid exponential phase at 52°C. Spore suspension was diluted and spread out in four petridishes with MMC medium. For UV irradiation a germicidal lamp (Philips) was used just 3" above the petridishes. After 2, 3, 4 and 5 minutes of irradiation the suspensions were diluted and plated for variable colony forming units (CFU). After 24h of incubation at 52°C colony forming numbers were counted. The experiments were carried out in dim red light and all precautions taken to prevent exposure of the incubated plates to direct light.

Osmotic Shock: Hyperosmotic shock, by increasing sucrose concentration, was given to both the mutant as well as wild strains of *T. vulgaris*, to test their colony forming ability. 0.1 ml of freshly prepared spore suspension in sterile normal saline was mixed with molten MMC medium containing different concentrations of sucrose (5 - 20% w/v) and incubated at 52°C. The numbers of viable colonies were recorded after 24h of growth.

RESULTS

The nal^r mutant was isolated on MMC medium containing varying concentration of nalidixic acid as mentioned in material and method (Figure 1, 2).

The constitutive resistance concentration (CRC) of this drug was found to be 50µg ml^{-1}. Several transfers of resist colonies on agar containing higher dose resulted in progressive increase in drug resistance showing a MBC (Maximum Bactericidal Concentration) 200µg ml^{-1} above which the colony growth was inhibited and phenotypic masking was observed. Lysis of colonies due to effect of the drug in the liquid medium was observed. Auxotrophy test on the MMS medium revealed that the mutant required asparagines as well as lysine (1mM each) for its optimal growth at 52°C.

The mutants (nal^r strain) possessed greater resistance to hyperosmotic shock (20% sucrose solution) (Table 1) as well as UV irradiation than the wild type (Table 2).

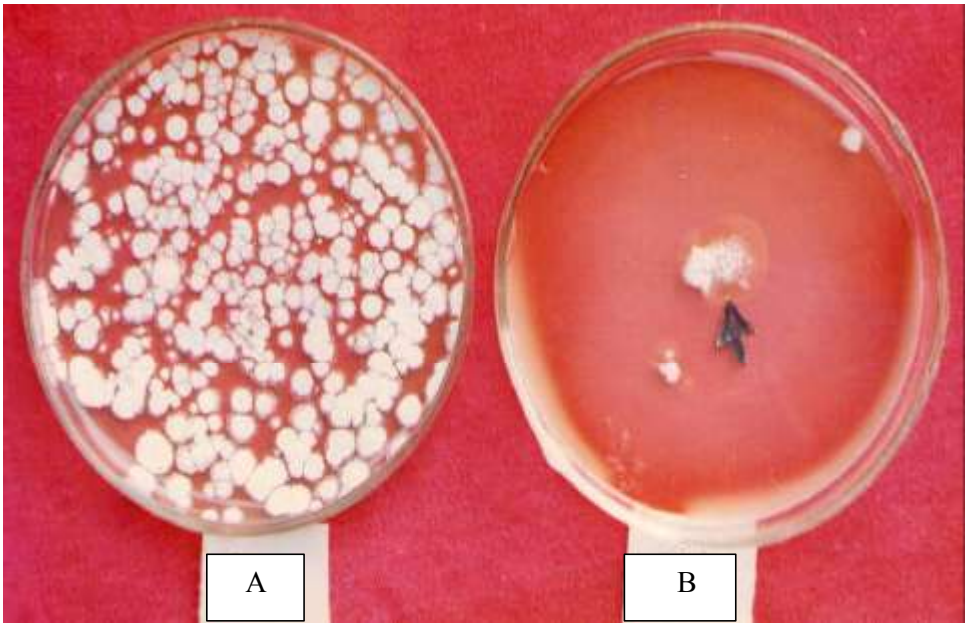

Fig. 1: (A) Wild type 1227 colonies growing on MMC. (B) Nalidixic acid resistant mutant colonies growing on CM

Table 1: Growth of wild type (1227) and nal^r strain viable cfu in the presence of different concentrations of sucrose

% of sucrose concentration in MMC medium	% cfu 1227	% cfu nal^r
3 (control)	98.4	98.8
10	28.0	48.0
15	10.0	26.0
20	-	15.0

Table 2: Effect of UV irradiation on wild type (1227) and nalidixic acid resistant mutant (nal^r), viable colonies forming units (cfu)

Time of exposure	1227 -uv	+uv	% survival (cfu)	nal^r mutant - uv	+uv	% survival (cfu)
2 min	178	128	76.22	211	178	84.36
3 min	189	74	39.18	168	79	47.02
4 min	148	25	16.80	145	48	33.10
5 min	158	11	07.08	150	35	23.30

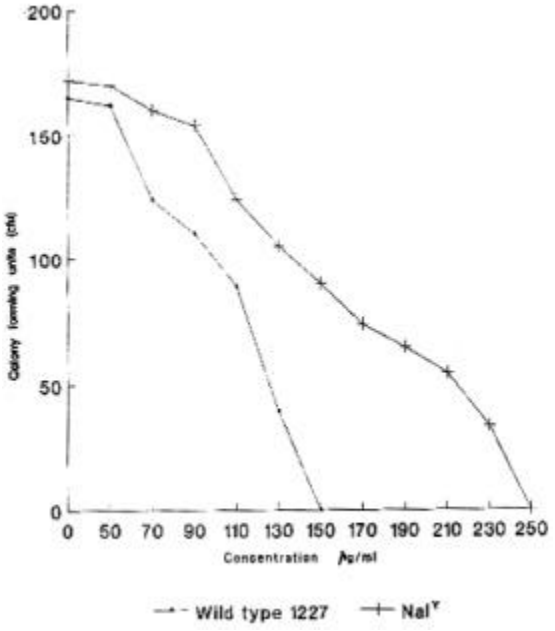

Fig. 2: Growth of wild type and nalidixic acid resistant mutant on varying concentrations of nalidixic acid

DISCUSSION

Bactericidal action of nalidixic acid in *E. coli* has been reported [1] where high doses of the drug inhibit both RNA and protein synthesis. It is also known to induce double strand break and DNA degradation [7, 11]. Nalidixic acid and other quinolones affect gyrase subunit A as well as topoisimerase IV in *E. coli*. Mutations in this gene (gyr A) and topoisomerase IV confer resistance to this drug in *Pseudomonas aeruginosa* PAO1 [2, 5, 11]. In case of *T. vulgaris* mutation in topoisomerase IV in addition to gyr A subunit provides additional resistance against this drug leading to double auxotrophy for asparagine and lysine.

Nalidixic acid and the gyrase inhibitors Norfloxacin/ Ciprofloxacin are known to cause alterations in cell envelope and membrane permeability in the colon bacteria [6, 12]. The high degree of resistance to hyperosmotic shock seen in the mutant of this thermophile could be due to alteration in membrane protein.

Induction of SOS repair system involving a number of genes rec ABC and lex A in *E. coli* due to quinolone drug has been observed [13, 14]. An efficient repair system is known to operate in *T. vulgaris* [15]. The view receives further support from the fact that the nal^r mutant presently studied is more resistant to UV than the wild strain 1227.

In conclusion the present study reveals that the trapping of DNA gyrase and topoisomerase IV by nalidixic acid probably leads to rapid inhibition of DNA synthesis, ceasation of growth and alteration in membrane structure and induction of SOS system also operates in this thermophile actinomycete. This observation shows analogy to earlier reported results due to DNA damage, replication leading to point mutation, chromosomal aberration and cell death [16].

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