Expression of Carotenoid Pigments of Haloarchaeal Cultures Exposed to Aniline

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ABSTRACT: The effects of exposure to aniline on growth and pigmentation in three haloarchaeal isolates from the Indian subcontinent—GUSF (MTCC 3265), from the estuarine saltpans of Goa, India; and GURFT-1 and GURFP-1, both from continental shelf sediments of the west coast of India—were studied. In nutrient-rich tryptone yeast extract medium containing 25% NaCl/crude salt, the growth of GUSF, measured as absorbance at 600 nm, was not affected significantly at all concentrations of aniline used [0.005%–0.04% (v/v)], whereas the growth phases of GURFT-1 and GURFP-1 were affected at concentrations $>$ 0.005%; the total yield, however, was nearly equal to the yield of cultures growing in the absence of aniline. GURFT-1 reached approximately 40% of total yield on the 7th day in the presence of 0.04% aniline, which declined thereafter. The pigmentation observed visually was completely abolished at concentrations of aniline greater than 0.01%. Spectral scans of acetone extracts of the pigment of each of the cultures exposed to concentrations of aniline $>$ 0.01% showed that (i) the bacterioruberin component of the pigments (absorbance in the range 390–500 nm) was completely abolished and (ii) the pigment component(s) shifted toward squalene and phytofluene derivatives (320–360 nm). This is the first report examining the effect of an aromatic pollutant such as aniline on the growth and pigmentation of haloarchaeal cultures. Environ Toxicol 20: 165–169, 2005.

Keywords: haloarchaea; aniline; pigmentation; carotenoids; bacterioruberin

INTRODUCTION

Haloarchaea, phylogenetically distinct and distant from Eukarya and eubacteria (Woese et al., 1990) occur in marine ecological niches. Haloarchaea have characteristic (C_{40}–C_{50} type) pigments that are involved in trapping photoenergy (Gibbons, 1974; Lanyi, 1997; Lanyi and Maeda, 1997) and in cells energetics as well as in the ecological niche (Tindall, 1988).

Anilines are toxic pollutants of major concern because of their extensive use in the chemical, agrochemical, pesticide, and dye industries (Meyer, 1981). In India aniline is widely found in effluents from organic chemical industrial concerns (Kumaran, 1993). The toxic effects of pollutants on the freshwater fish *Oreochromis mossambicus,* the crustacean *Moina micrura,* the worm *Branchiura sowerbyi,* marine bottom fish species, and the grass shrimp *Palaemonetes pugio* have been monitored for loss of appetite, impairment of reproduction, production of hepatic lesions, deformation of organs and/or mortality (Rayburn et al., 1996; Lee and Page, 1997; Myers et al., 1998; Bhunia et al., 2003). The response of haloarchaeal cultures to toxic compounds such as aniline in growth and pigmentation is unknown. Hence, to determine the effect of aniline on a group of haloarchaeal bacteria, we used three halophilic archaea: GUSF (MTCC 3265) isolated from the salt pans of Goa, India (Sequiera, 1992; Khandavilli et al., 1999; Khandavilli, 2001; Braganca, 2003), and GURFT-1 and GURFP-1, both isolated from the continental shelf region off Gujarat, India (Raghavan and Furtado, 2004). Each of the three haloarchaeal cultures had an orange-red pigmentation, reflecting the presence of bacterioruberins and carotenoids.

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MATERIALS AND METHODS

Microbial Cultures and Maintenance

Cells of GURFT-1 were gram-negative rods, whereas those of GURFP-1 were gram-negative cocci. The pleomorphic nature of the gram-negative rods of GUSF was in agreement with previous results (Aguiar and Furtado, 1996).

GUSF, GURFT-1, and GURFP-1 were maintained in a nutrient-rich tryptone yeast extract (TYE) medium containing NaCl/crude salt (250 g/L) at a pH adjusted to 7 with 1M NaOH (Steensland and Larsen, 1968), hereafter referred to as NTYE, and the medium was solidified with 1.5% agar (w/v).

Monitoring of Growth and Pigmentation

The growth and pigmentation of GUSF, GURFT-1, and GURFP-1 were studied by culturing each separately in 250-mL flasks containing 50 mL of NTYE and aniline in 0.005%, 0.01%, 0.02%, and 0.04% concentrations (v/v). Flasks containing only 50 mL of NTYE without aniline or the respective culture(s) served as controls. All flasks were incubated at ambient temperature (28°C–30°C) and 150 rpm. In each culture the development of an orange-red pigmentation was followed visually, and increases in absorbance were followed with a spectrophotometer (UV-6401, Shimadzu) at 600 nm. Each treatment was carried out in sets of three flasks 3 times.

Spectral Analysis of Pigments

Stationary-phase cells of GUSF, GURFT-1, and GURFP-1 grown for 6 days in NTYE were individually harvested by centrifugation at 8000 rpm (REMI, India), washed, resuspended in a minimal amount of a solution of 20% NaCl and acetone (1:1), and subjected to sonication in a Labsonic (Braun, USA) sonicator, using a medium-sized probe at 85 Hz and a pulse of 6 s for 5 min. The acetone fraction containing the pigments was freed of debris and scanned between 300 and 700 nm.

RESULTS AND DISCUSSION

Each of the GUSF, GURFT-1, and GURFP-1 haloarchaeal cultures grew optimally in TYE containing 25% NaCl with a 1-day lag but failed to grow in TYE without any NaCl.

In the presence of up to 0.01% aniline, growth of GUSF and GURFP-1 was identical to the respective control cultures growing without aniline. All three cultures grew exponentially after the first day of inoculation and reached the stationary phase by the fourth day (Fig. 1). Aniline up to 0.005% had no effect on the onset of growth and the subsequent growth phases of GURFT-1 and GURFP-1. Interestingly, the growth rate in presence of 0.005% aniline, which is less than that of the cultures growing without aniline, was not affected by any further increase in aniline concentration from 0.01% to 0.04% (Fig. 1). Doubling of aniline concentration, however, extends the stationary phase of GURFT-1 by a day and affect cell yields adversely (Fig. 1).

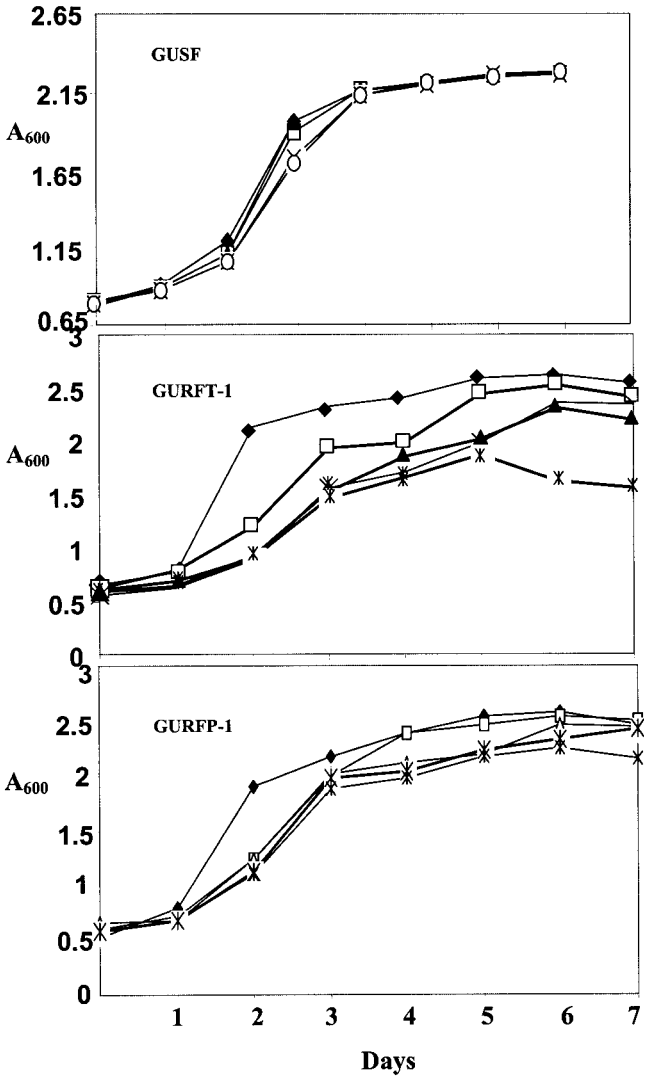

Fig. 1. Growth of haloarchaeal cultures in NTYE containing aniline: —◆— 0%, —□— 0.005%, —▲— 0.01%, —×— 0.02%, —○— 0.04%.

After 7 days of growth in NTYE, the number of cells was 5.30×10^{10} for GUSF, 5.95×10^{10} for GURFT-1, and 5.75×10^{10} for GURFP-1 (Fig. 2). The presence of aniline in a 0.005%–0.04% concentration did not adversely affect the growth of GUSF and GURFP-1 and yielded a total of $5.30–5.35 \times 10^{10}$ and $5.02–5.75 \times 10^{10}$ cells, respectively. At a concentration of 0.04% aniline, GURFT-1 yield decreased to 3.64×10^{10} cells/mL as against its yield of 5.95×10^{10} cells/mL in the absence of aniline.

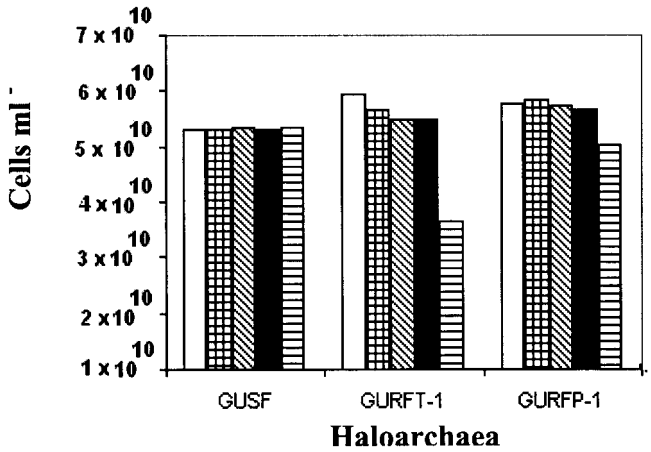

Fig. 2. Total yield of haloarchaeal cultures on the seventh day of growth in NTYE and aniline: □ 0%, ▦ 0.005%, ▧ 0.01%, ■ 0.02%, ▤ 0.04%.

The assimilation and degradation of aniline in eubacterial cultures in *R. erythropolis* AN-13 were reported by Aoki et al. (1983) and in *Rhodococcus* mutant strain AM144 by Janke and Ihn (1989). As yet, there have been no published reports on the response of haloarchaea to aniline.

A 0.01% concentration of aniline delayed the pigmentation of cells by 1 day in GUSF and GURFT-1 and by 2 days in GURFP-1. Delay in pigmentation and in the formation of isoprenoid compounds in *Halobacterium cutirubrum* and *Halobacterium halobium* cells growing in glycerol, which was attributed to nutritional control, was reported previously (Gochnauer et al., 1972).

Acetone extracts of GUSF, GURFT-1, and GURFP-1 cells pregrown in NTYE without aniline were orange-red in color and had peaks at 350, 370, 388, 425, 468, 495, 528, and 596 nm, 342, 465, 495, and 528 nm, and 342, 465, 496, and 526 nm, respectively. The peaks with absorption maxima at 370, 388, 494 and 527 nm corresponded to those of bacterioruberin (Kushwaha et al., 1974), the peak at 465 nm to neurosporene (Kelly et al., 1970), the peak at 468 nm to lycopene (Kelly et al., 1970), and the peak at 425 nm to β-carotene (Kelly et al., 1970). The archaeal pigment profiles thus confirmed the C_{30}–C_{40} haloarchaeal pigments characteristic of GURFT-1 and GURFP-1 and of GUSF and corroborated our earlier findings (Raghavan and Furtado, 2004).

With an aniline concentration up to 0.01%, the profiles of the pigments formed were identical to those of the control (Fig. 3). For GUSF, these results corroborated our earlier findings on the tolerance of the culture to crude oil and constituent hydrocarbons (Raghavan and Furtado, 2000). At concentrations above 0.01%, none of the three cultures developed the characteristic orange-red pigmentation, even after 5–6 days. However, at these concentrations there was a significant increase in absorbance at 600 nm, suggestive of growth (Fig. 1), which was supported and confirmed by the total viable cell counts (Fig. 2). An increase in the concentration of aniline therefore delayed pigmentation and exerted a selective inhibitory effect on pigmentation at concentrations above 0.01% without affecting the growth of any of the three cultures.

Above a concentration of 0.01%, total bacterioruberin decreased from 0.027 to 0.018 μg/g protein in GUSF, from 0.028 to 0.004 μg/g protein in GURFT-1, and from 0.006 to 0.0045 μg/g protein in GURFP-1. Total bacterioruberin content was calculated using the formula

$$X = A.y/(E^{1\%}.100),$$

where X is the bacterioruberin content, A is the absorbance, y is the path length (1 cm), and $E^{1\%}$ is the extinction coefficient 2540 (for bacterioruberin; Kushwaha et al., 1972).

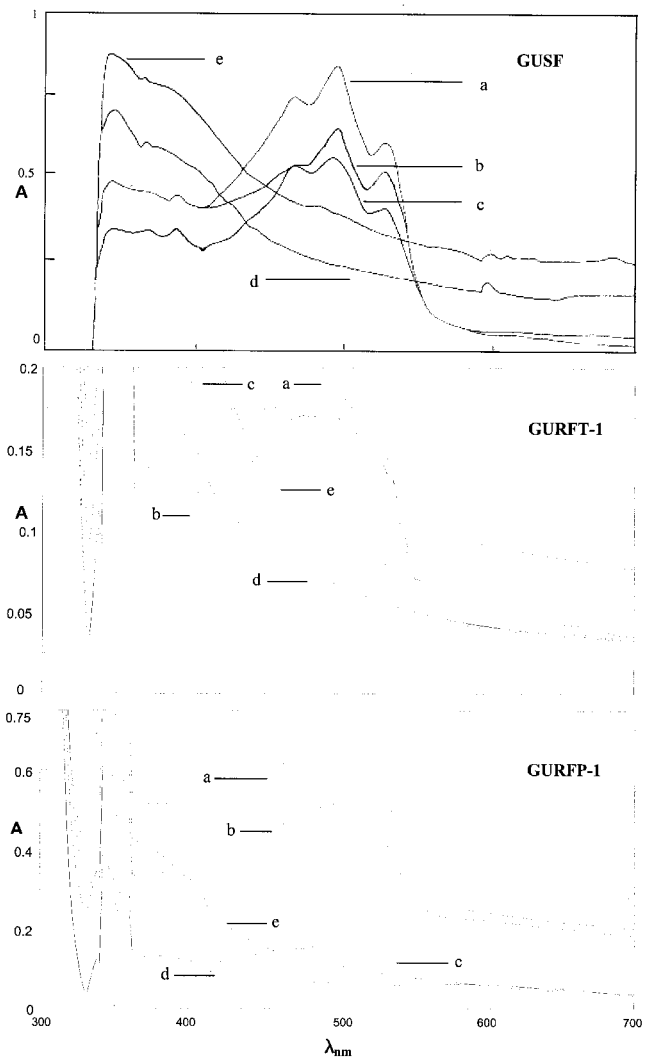

Fig. 3. Pigment profiles of haloarchaeal cultures grown in NTYE and NTYE with aniline: (a) 0%, (b) 0.005%, (c) 0.1%, (d) 0.02%, (e) 0.04%.

All three cultures showed complete abolition of peaks in the range of 390–500 nm with a concomitant increase in absorbance at 388, 344, and 342 nm, and in the GUSF culture a peak appeared at 590 nm. The appearance of a broad band between 320 and 360 nm corresponds to phytofluene derivatives (Jensen et al., 1958). This effect is comparable to that of glycerol on the pigmentation of *Halobacterium cutirubrum* (Gochnauer et al., 1972). Higher concentrations of aniline thus induce increased production of phytofluene and related compounds, possibly as a stress-abating response. The peak at 590 nm is suggestive of rhodopsin, known to be induced during growth in subanoxic conditions (Tindall, 1988).

Haloarchaeal carotenoid pigments, namely, bacterioruberin and other accessory pigments, are known to be involved in membrane energetics (Lanyi, 1997; Lanyi and Maeda, 1997), in addition to shielding cells from damage from UV and free radicals (Salto et al., 1998). It is noteworthy that in the present study the cells continued to grow at aniline concentration greater than 0.01% but were devoid of the bacterioruberin component of the pigment. The abolishment of the bacterioruberin peak was accompanied, however, by increased production of squalene derivatives with absorbance peaks below 300 nm, indicating that at aniline concentrations above 0.01%, the haloarchaeal cells shifted their cellular physiology from synthesizing bacterioruberin to synthesizing squalenes/phytofluene.

A delay in the onset of pigmentation possibly could affect membrane bioenergetics. Lipophilic compounds such as aniline and benzene are known to increase the amount of saturated fatty acids in eubacterial membranes (Rosas et al., 1980; Sikkema et al., 1995). Because haloarchaea do not possess fatty acids, an increase in phytofluene in the presence of aniline at a concentration of 0.01% or greater also could be a response mechanism of haloarchaea to aniline.

Furthermore, the loss/impairment of visual pigmentation without a concomitant effect on growth makes haloarchaeal cultures candidates for toxicity studies using different classes of hydrocarbon pollutants. This is the first scientific attempt at examining the effect of aniline on the growth and pigmentation of haloarchaeal cultures.

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