# CHEMTAXIS IN SPIROCHAETA AURANTIA: A POSSIBLE CATION EFFECT. SCREENING FOR POTENTIAL CHEMOREPELLANTS

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## INTRODUCTION

The aim of this investigation was to develop a more thorough understanding of chemotaxis in Spirochaeta aurantia. S. aurantia is a true bacteria that possesses a morphology and form of locomotion unique to all other orders of bacteria. Many investigators have attempted to understand the full mechanism behind spirochete motility, but much is left to speculation. Chemoreception in S. aurantia as well as other bacteria is of particular interest; a better understanding of chemoreception and its tie with motility would provide us with information regarding a very primitive form of behavior. The mechanism by which bacteria distinguish chemical differences in their environment has been well characterized.

Cations are known to play important roles in osmoregulation and in the maintenance of enzymes and other macromolecules during anabolic and catabolic processes in bacteria. One goal of this study was to provide preliminary findings on the effects that different salts at varying concentrations have on chemotaxis in S. aurantia. A second goal of this study was to develop an assay that would determine if a substance could serve as a chemorepellent to S. aurantia. Ideally, these findings would provide us with a better understanding of the ecology of S. aurantia as well as provide us with valuable information leading to a thorough understanding of spirochete behavior.

## MATERIALS AND METHODS

Bacterial strain and growth conditions. Spirochaeta aurantia strain M1 was the organism of interest throughout this investigation. S. aurantia was cultured in glucose-trypticase (BBL)-yeast extract media according to Greenberg and Canale-Parola (1977); the final addition of 1 M postassium phosphate buffer was omitted. Batch cultures were grown in 50 ml quantities at 30°C; 3 ml stock cultures were maintained at room temperature by transferring aseptically 0.1 ml of 40 hr culture approximately every other day.

Migration studies. The procedure for chemotaxis outlined by Breznak and Canale-Paroli (1972) was followed with little variation. The CT media contained 0.2% glucose in addition to trypticase and yeast extract to provide maximal migration rates. Various salts were diluted from a stock 2.0 M solution into 20.0 ml of CT media to give the desired final concentration. After allowing the agar to set, each migration plate was spotted with late log phase (40-44 hr) culture. After allowing 3 hrs for the inocula to be absorbed into the solid agar, the plates were incubated at 30°C. At various time intervals (every 12 hrs), the plates were removed from the incubator and the migration rings measured. Migration rates were calculated by dividing migration distance by time.

Measurement of growth. Flasks (125 ml) containing 50 ml GTY media at the desired salt concentrations were inoculated with S. aurantia and incubated at

30°C for 60 hrs. Approximately every 6 hrs a 2.5 ml subsample was removed aseptically from the culture flask and measured at 660 nm on a Gilford 2600 spectrophotometer. $Log_{10}$ absorbance was plotted against time. Mean doubling time was calculated according to Stanier et al. (1976) by taking the reciprocal of k, where,

$$k = \frac{\log_{10} \text{Abs. } 2 - \log_{10} \text{Abs.} 1}{(0.301)\ (\text{Time } 2 - \text{Time } 1)}$$

Linear regressions were performed on the data points comprising the exponential phase of growth. Points 1 and 2 indicate two hypothetical times during that phase.

Photographic studies of spirochete behavior. Cells were grown in 20 ml of GTY media at 30°C. After 40 hrs the cells were centrifuged at 3000 g for 10 min and resuspended in 10 ml GTY media. One-to-five dilutions were made into respective culture tubes to give the desired final concentrations. Diluting 1:5 reduced spirochete numbers by 60% and gave a per-microscopic-field count of approximately 100 cells. At 20 microliter drop a suspension was placed on a large, clean coverslip and viewed at 400X with a Zeiss inverted scope equipped with a darkfield condensor and mercury lamp. Four sec exposures were made with the microscope focused at the coverslip-droplet interface on Kodak Plus-X film. After processing, the negatives were slide-mounted and projected in a darkened room. A total count was made; then counts of those spirochetes that appear to be flexing (appear as blurs due to long exposure) or translating (appear as streaks). The quotient of translators/total X 100% yielded the percent of spirochetes not flexing.

Screening for potential chemorepellents. The chemical-in-plug method developed by Tso and Adler (1974) was employed in initial experiments. Spirochete cells were harvested after 44 hrs of incubation at 30°C. Approximately 30 ml of cell suspension were required per assay; this quantity was centrifuged for 10 min at 3000 x g. The supernatant was carefully removed and the remianing pellet resuspended in 0.01 M potassium phosphate buffer containing $2 \times 10^{-4}$ M L-cysteine hydrochloride. Note that EDTA was not used in any of the solutions; EDTA is supposedly lethal to S. aurantia. The spirochete cells were centrifuged once again. The pellet was resuspended once more in potassium phosphate buffer plus L-cysteine hydrochloride. The optical density of the resulting suspension was noted; a predetermined volume of this suspension was diluted into 20.0 ml chemotaxis buffer containing 0.3% agar (at 50°C) to give a final optical density range between 0.1 and 0.3. After allowing the test plate to cool for 5 min, the test chemical plugs were placed carefully in the sloppy agar. The plates were allowed to develop for 30 min and the clearing zones, if any, were measured.

## RESULTS

Migration and growth studies. Figure 1 illustrates the effect that a range of NaCl concentrations has on the chemotaxis of Spirochaeta aurantia. In this particular series, note that the migration ring reaches a maximum diameter at 25 mM whereas the migration rings are much smaller in diameter at concentrations above and below 25 mM. As evident by the malformed, diffuse migration ring, chemotaxis at 60 mM appears to be severely impaired. In two later replicates of this same experiment, the migration rings were at a maximum again at 25 mM NaCl, but the diffuse migration ring occurred at 50 mM,

indicating a possible dilution error in the previous trial. To test whether the chloride ion was affecting chemotaxis, migration plates containing identical concentrations of $Na_2SO_4$ were monitored over a 60 hr period. Although slight, $Na_2SO_4$ did stimulate migration at 10 mM. The diffuse migration ring occurred this time at 25 mM, one half the concentration required for NaCl. KCl appeared to have the same effects on chemotaxis as NaCl. $CaCl_2$ markedly stimulated migration at 10 mM but reduced migration rate by more than 90% at 25 mM. Figure 2 shows migration rate plotted against concentration for the aforementioned salts. Figure 3 shows mean doubling time as a function of NaCl and KCl concentration.

Photographic studies of spirochete behavior. Table 1 shows the percentage of spirochetes that are translating (not flexing) in GTY media containing various concentrations of NaCl and KCl. Two estimates were made of each slide so as to reduce the subjectivity of this study; an average differed by no more than 5% of the estimates. These data represent the behavior of S. aurantia when they were transferred from their culture media to culture media containing various salt chemorepellants was attempted. A chemotaxis viewing chamber was constructed by gluing broken end pieces of a microscope slide with silicon cement to a whole slide. A coverslip was bridged across the end pieces. An inoculum of 40 hr S. aurantia culture was injected into the chamber. A capillary tube containing 1 M test chemical was then inserted into the droplet, and the mouth of the capillary tube was watched through an inverted phase scope at 400X. Theoretically, the spirochetes could be observed directly with this method; a chemorepellent could be tested through direct observation. Phenol, methanol, propanol, isopropanol, and citric acid were tested using this method; the spirochetes were neither attracted nor repelled. S. aurantia was not attractd towards a capillary tube containing 1 M D-glucose.

## DISCUSSION

Chemotaxis in S. aurantia: a possible cation effect. Migration in S. aurantia is stimulated at 10 mM concentrations of $CaCl_2$ and $Na_2SO_4$ and at 25 mM concentrations of NaCl and KCl. But an increase in salt concentration leads to a decrease in migration until, at some critical concentration, the spirochetes seem to lose their ability to distinguish different levels of glucose (Figs. 1 and 2). We may rule out the possibility that the migration studies are showing differences in growth rate due to varying concentrations of salt. Figure 3 shows that the growth of S. aurantia remained relatively constant at NaCl and KCl concentrations greater than 25 mM. However, microscopic examination of the spirochete cells used during the growth studies revealed cells as much as three times longer in the 50 and 75 mM cultures than those grown at lower concentrations of NaCl and KCl. Thus, perhaps growth remains relatively constant over increasing salt concentration, but cell division is somehow delayed. If this is the case, one must consider the effect that increased cell length would have on the motility of S. aurantia. In the migration studies one would hypothesize that the chemosensor mechanism is somehow impaired at 50 mM concentrations of NaCl and KCl. This is evident by the presence of a diffuse migration ring, indicating that the motility system of S. aurantia is still operational, but its ability to determine which direction to travel is lacking. Photographic studies of spirochete behavior showed the contrary. According to Table 1, S. aurantia maintained or increased translational motility with increasing salt concentration. One must

take into account that this study demonstrated the immediate effect that increasing salt concnetration had on behavior rather than the long-term effect as demonstrated in the migration plate studies. Thus more experimentation is needed to determine the effect that cations have on chemotaxis in S. aurantia and the possible effects of cations on growth (cell division) and motility.

Screening for potential chemorepellents. None of the chemicals tested in this study proved to be effective chemorepellents. Sodium acetate at $3 \times 10^{-1}$ M did produce a slight clearing zone, but this may have been an artifact. The search for the first chemorepellent continues. Rather than testing thousands of chemicals at random, we attempted to selected likely candidates such as substances known to be metabolic end products in S. aurantia or substances known to effect negative chemotaxis in E. coli. There is a need to find a more rapid method of screening chemorepellents. The method developed by Tso and Adler (1974), although sensitive, is quite time consuming.

TABLE 1
SPIROCHETE BEHAVIOR IN VARIOUS CONCENTRATIONS OF NACL AND KCL

| Salt | Percent not flexing |
|---|---|
| Control | 84.3 |
| NaCl 10 mM | 87.2 |
| 25 mM | 89.0 |
| 50 mM | 88.5 |
| 75 mM | 94.8 |
| KCl 10 mM | 92.0 |
| 25 mM | 93.7 |
| 50 mM | 93.3 |
| 75 mM | 93.3 |

Screening for potential chemorepellents. Table 2 gives a listing of six chemical tested for chemorepellency. None of the chemicals caused distinct clearing zones as described by Tso and Adler (1974). Sodium acetate at $3 \times 10^{-1}$ M did exhibit a slight clear sone surrounding the plug; however, this was 2 hrs after the plug was set into the sloppy agar. Glucose, at $1 \times 10^{-1}$ M, caused a faint accumulation of spirochetes after 2 hrs.

TABLE 2
TESTING POTENTIAL CHEMOREPELLENTS WITH THE CHEMICAL-IN-PLUG METHOD

| Test Chemical | Concentration range (M) |
|---|---|
| L-alanine | $1 \times 10^{-1} - 1 \times 10^{-5}$ |
| Ethanol | $1 \times 10^{0} - 1 \times 10^{-4}$ |
| D-Glucose | $1 \times 10^{-1} - 1 \times 10^{-5}$ |
| Indole | $1 \times 10^{-1} - 1 \times 10^{-5}$ |
| L-phenylalanine | $1 \times 10^{-1} - 1 \times 10^{-5}$ |
| Sodium acetate | $3 \times 10^{-1} - 3 \times 10^{-4}$ |

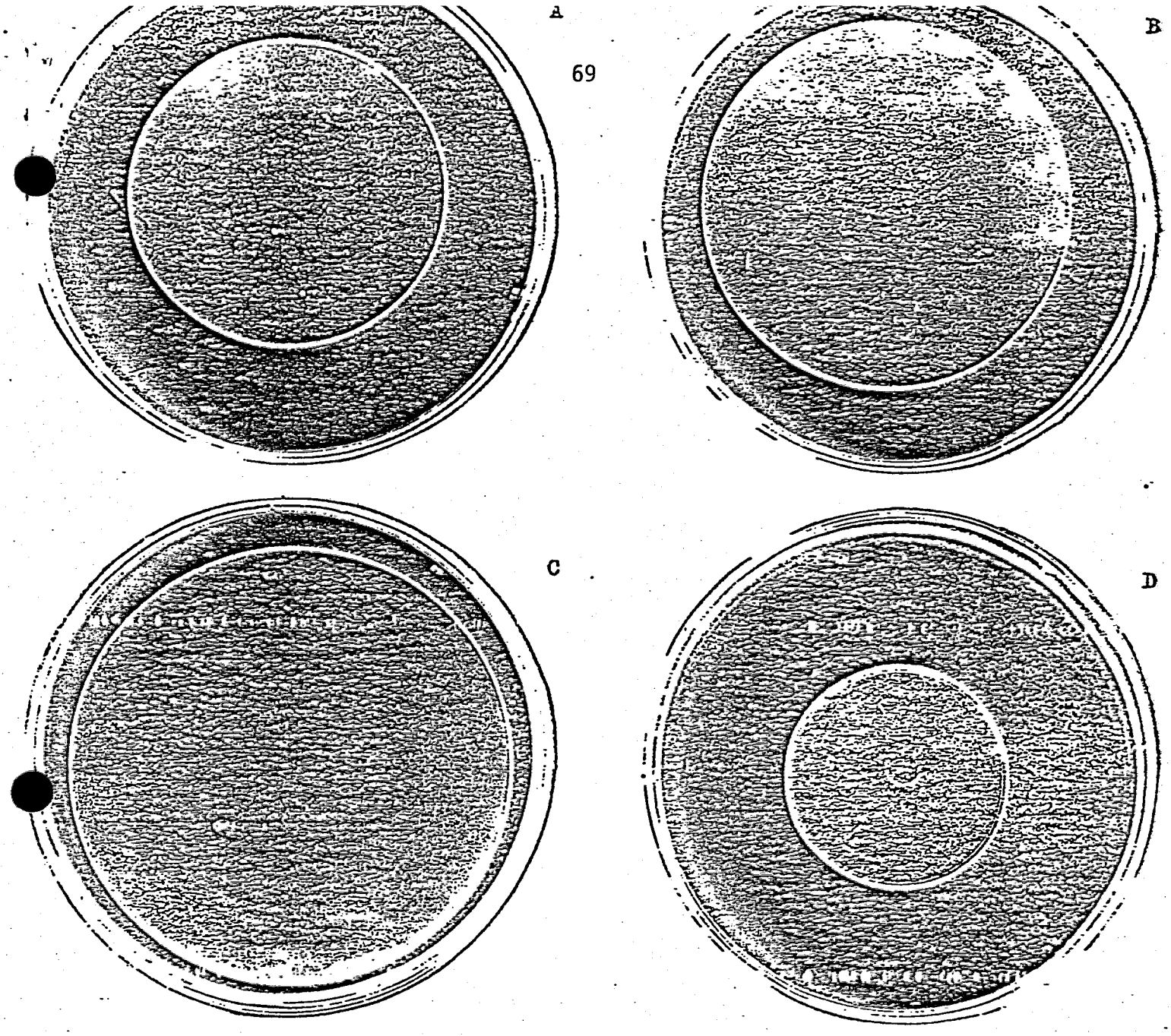


Fig. 1. Plates A through E contain glucose-limited media with increasing concentrations of NaCl: A, 0 mM; B, 10 mM; C, 25 mM; D, 50 mM; and E, 60 mM. Each plate was spotted with a 10 microliter inoculum of S. aurantia and incubated for 60 hours at 30° C. The spirochetes exhibit positive chemotaxis as they utilize glucose and migra outward in pursuit of higher glucose concentrations.

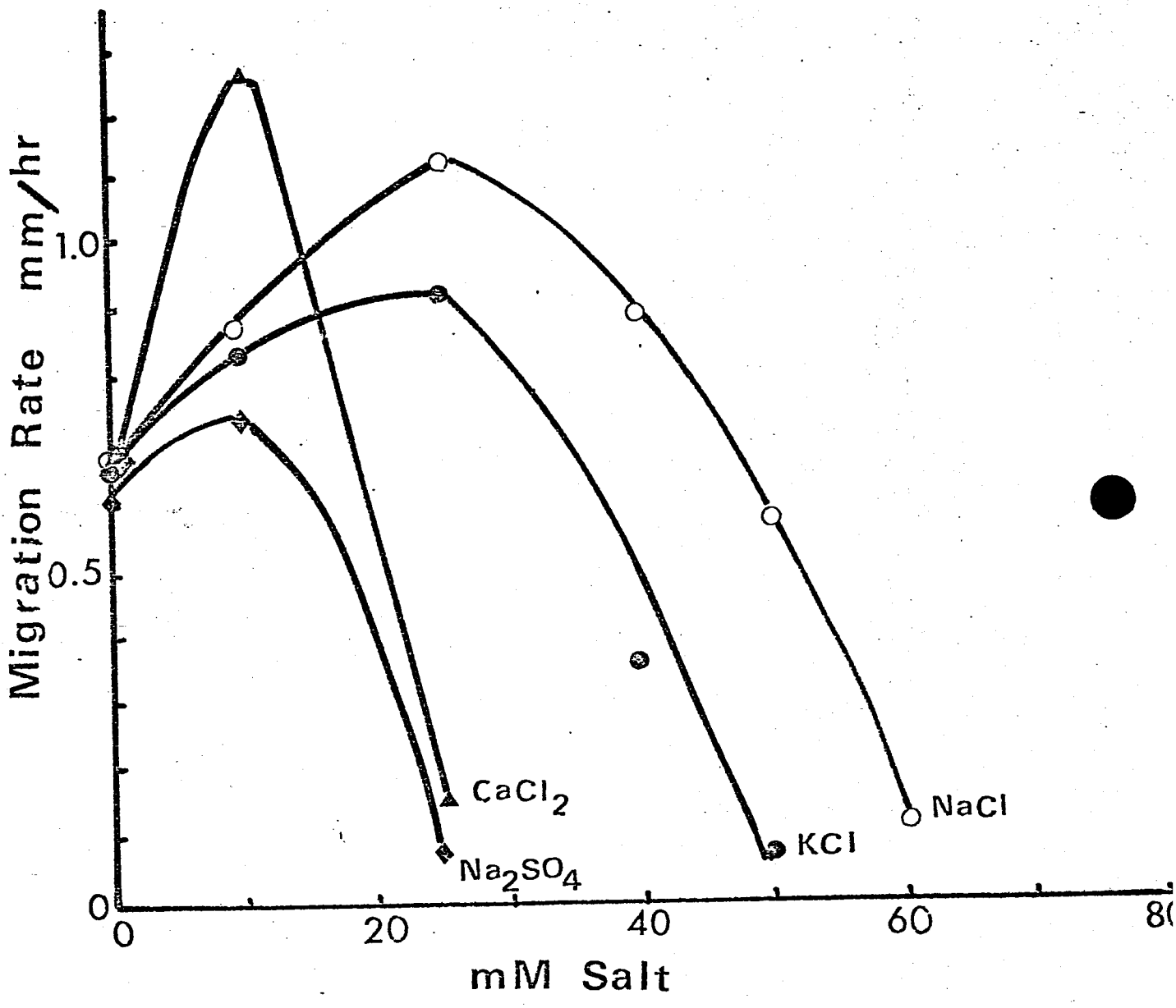


Figure 2. Migration rate of S. aurantia as a function of salt concentration

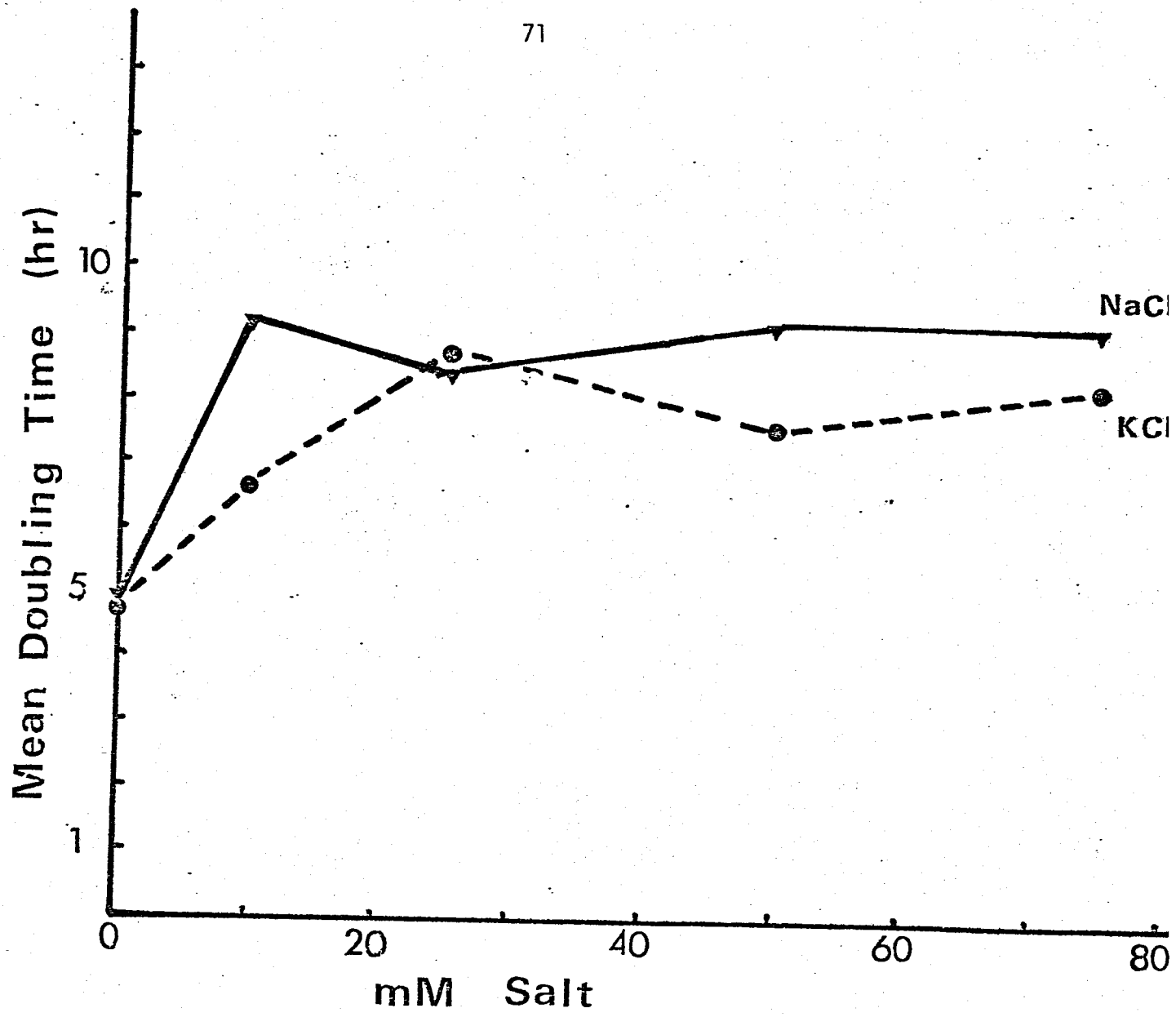


Figure 3. Mean doubling time of S. aurantia as a function of salt concentration.

# ISOLATION OF MARINE TOLUATE-OXIDIZING BACTERIA

Nicholas Santoro

## MATERIALS AND METHODS

Bacterial strains. The two TOL plasmid-containing organisms that were used in this study were Pseudomonas arvilla (PaW) and Pseudomonas putida (arvilla) mt-2.

The organisms, designated XF8, SF9 and XF15 were isolated from the Great Sippewissett Salt Marsh, Woods Hole, Mass. Initially, two types of marsh plots were used to isolate the organisms. One type of marsh plot was a heavily fertilized plot (designated XF) and the second was an unfertilized, control marsh plot (designated C). Samples of the marsh plots were taken from only the top 2 cm of each plot. These 2 cm core samples were made using a cork borer. Two XF plots were sampled and 2 C plots were sampled. Three core samples were taken from each individual plot. The core samples were collected in large beakers designated either XF or C, and the contents in each beaker were then mixed together with a spatula. A sample weighing 1 gram was then removed from each beaker and placed in 250 ml Erlenmeyer flasks containing 100 ml of a modified Baumann and Baumann (1981) medium containing 5 mM m-toluate (MBT medium). The flasks were incubated on a shaker for 4 days at 30°C. After incubation, 1 ml aliquots were taken from the above flasks and diluted $10^1$, $10^2$ and $10^3$ using 9 ml artificial seawater (ASW) dilution blanks. Next, 0.1 ml was removed from each dilution blank and transferred to MBT agar medium using the spread plate technique.

After incubation, the plates were examined with the aid of a stereo dissecting microscope. Based on differences observed in the morphological characteristics of the colonies on the above plates, 15 isolates were obtained from the XF plots and 6 from the C plots. Two colony types were readily distinguished, they produced a diffusable pigment which spread through the surrounding agar. One colony type produced a brownish-black pigment, while the second colony type produced a yellow pigment. Neither colony type fluoresced when exposed to long wavelength ultraviolet light. Spread plates designated XF contained both types of pigment producing colonies. Only the brownish-black pigment-producers were observed on the C plates.

As many different colonies as possible were picked from the XF and C spread plates. The colonies were streaked on fresh MBT plates and incubated for 48 h at 30°C. This process was repeated until the 15 XF isolates and 6 C isolates were purified. Out of the above XF isolates, all but XF8 XF9 and XF15 were discarded because they grew either as well or better on MB agar (minus m-toluate) as on MBT agar. These colonies also did not turn liquid MBT shake tubes turbid. In comparison, XF8, XF9 and XF15 grew very poorly on MB agar (minus m-toluate) when compared with growth on MBT agar. These 3 isolates also produced turbid MBT liquid shake tubes after incubation for 48 h at 30°C. The isolates XF8, XF9 and XF15 are yellow pigment-producing colony types. They are given different designations due to the different colonial appearance that each displays on MBT agar.

All of the C-isolates were discarded because they either grew as well or better on MB agar minus m-toluate as on MBT agar. Some of the C isolates