Buffering Capacity of Pigmented and Nonpigmented Strains of Serratia marcescens

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The pigmented strain Serratia marcescens ATCC 274 had a higher buffering capacity and a higher membrane H⁺ conductance than S. marcescens GP, a spontaneous nonpigmented mutant of ATCC 274. The data suggest that mutations which apparently affect only the synthesis of a secondary metabolite can modify buffering capacity and passive H⁺ conductance.

Prodigiosin is a red pigment synthesized by biogroups A1 and A2 of Serratia marcescens. It seems clear that the biosynthesis and cellular localization of prodigiosin are strongly related to the physiology of the bacterial envelope (14, 20). Paruchuri and Harshy (14) suggested that color variation associated with prodigiosin is related to a variation of surface antigens that may serve as a way to evade the immune system. Other authors suggested that nonpigmented strains are better receptors of R factors than pigmented ones, which could be an advantage in clinical environments (2, 15, 16, 19).

Williams et al. (21) studied the influence of some environmental conditions on prodigiosin production by suspensions of nonpigmenting cells of S. marcescens, but their efforts to investigate the effect of pH on pigment formation failed, since the suspensions themselves provided such effective buffering. Irrespective of whether the pH of the nonpigmenting cells was acid or alkaline, the cultures rapidly approached a pH of 8.0 to 8.3 and maintained this range throughout incubation for 7 days.

Some authors measured the buffering capacity of gram-positive bacteria (1, 5, 6, 9, 18, 23). However, there are several technical problems associated with measurement of buffering capacity of gram-negative bacteria. Krulwich et al. (6) showed that the method of permeabilizing Escherichia coli cells strongly influences internal buffering capacity (Bᵢ) measurements. The permeability of gram-negative bacteria to cations is greatly reduced by the outer membrane. We have treated cells with EDTA to make them permeable to protons, on the basis of earlier observations by Leive (7) and Hirota et al. (4). Rosen (17) found a low membrane conductance to protons of anaerobic suspensions of E. coli at about pH 7, but we have not found any direct estimates of H⁺ conductance for gram-negative bacteria over a wide range of external pH in the literature. In this article, we provide a quantitative description of membrane conductance to H⁺ (C⁺m) of two strains of gram-negative bacteria over a wide range of external pHs (3.84 to 7.58).

The aim of the present study was to compare the buffering capacities and the membrane H⁺ conductances of two strains of S. marcescens that differ only in a precursor(s) of prodigiosin (12).

S. marcescens GP is a nonpigmented mutant of S. marcescens ATCC 274 (12) belonging to the same biogroup, A2a, as the wild-type strain (3). Both strains were grown aerobically with shaking to the early stationary phase (8 to 10 h) in complex medium containing meat peptone (0.5%, wt/vol), casein peptone (0.5%, wt/vol), yeast extract (0.3%, wt/vol), sodium chloride (0.5%, wt/vol), and glycerol (0.8%, vol/vol) and then washed three times with 300 mM KCl. The washed cells were centrifuged, resuspended in 20 ml of 300 mM KCl, and placed in a reciprocating shaker maintained at 37°C and EDTA (3 mM final concentration, pH 6.2) was added. Five minutes later, the cells were diluted 10-fold with the same type of medium that was used to wash them and centrifuged at room temperature. The pellet was resuspended in 300 mM KCl to a final concentration of 5 to 10 mg of cell protein per ml. Protein content was determined by the method of Lowry et al. (8).

The buffering capacity and membrane conductance to H⁺ of S. marcescens ATCC 274 and GP were measured by the method of Maloney (9). Experiments were conducted on 7-ml samples of cell suspensions, in 10-ml glass vials, which were stirred magnetically at room temperature. The pHs of these suspensions were between 6.4 and 6.8. Valinomycin (Sigma Chemical Co.) was then added (10 μM final concentration) as small volumes of concentrated stocks in acetonitrile; final acetone concentrations did not exceed 0.2%. The cells were allowed to equilibrate for about 2 h with intermittent mixing. Immediately before the assay, 0.23 ml of freshly prepared carbonic anhydrase (Sigma) (20 mg ml⁻¹ in 300 mM KCl) was added. Vigorous mixing with a small magnetic flea was begun after insertion of the pH electrode. After 5 to 10 min, an acid pulse was given, usually as a 50- to 150-μl portion of 100 mM HCl in 300 mM KCl, and changes in external pH were recorded for 3 min.

When assays were performed below or above pH 6.8, an initial pH adjustment was made during the 2-h preincubation period. In these cases, 50-μl quantities of 100 mM HCl or KOH (in 300 mM KCl) were added at intervals of about 20 min until the desired pH was attained.

The pH recordings were analyzed graphically as described elsewhere (9–11, 18). The difference between initial pH and final equilibrium pH was used to estimate total buffering capacity (Bᵢ). At 20-s intervals, the difference between measured pH and final equilibrium pH was plotted on a logarithmic scale against time. Back extrapolation gave the value of the pH overshoot at time zero (pHᵢ). The difference between initial pH and pHᵢ was used to estimate external buffering capacity (Bₑ). Because of the mixing artifact and because the half time for response of the pH recording system was 2 to 3 s, pH changes during the first 15 s were not used in these plots.
The difference between $B_t$ and $B_n$ was not significant when all pHs were included (Fig. 1). The experiments used the half time of the approach to the final values. Measurements of $B_t$, $B_n$, and the observed half time of the approach to the final equilibrium were used in the calculation of $C^{\text{H}_2}_m$ (9).

Estimates of both buffering capacity and membrane conductance to $\text{H}^+$ were dependent on the $\text{pH}$ attained during preincubation. For example, when titration was started at $\text{pH}$ 7 and the $\text{pH}$ was adjusted to 4, the measurements at the lower $\text{pH}$ were not significantly different from estimates obtained when titrations were performed with cells first equilibrated at $\text{pH}$ 4. This overlap also indicates that the preparations remained stable during the several hours required for collection of data.

Figure 1 summarizes measurements of $B_t$ and $B_n$ as a function of $\text{pH}$ for each strain studied. Over this $\text{pH}$ range, there were considerable changes in both $B_t$ and $B_n$ with a marked tendency for each to increase as the $\text{pH}$ became more acidic. The individual estimates of $B_t$ are not shown; instead, they were calculated as the difference between the two smooth curves that described the behavior of $B_n$ and $B_t$. These smooth curves were obtained from a polynomial regression. Several studies of buffering capacity of bacterial cells have suggested that $B_t$ and $B_n$ values vary markedly between species. The most important finding of this study was that buffering capacity and membrane conductance to protons were quite different from strain to strain. Our data confirm the common belief that $B_n$ is a significant proportion of $B_t$; thus, changes in the effective $\text{pH}$ (pH$_{\text{eff}}$) of a dense cell suspension would not be completely reflected in changes in the cytoplasmic $\text{pH}$ because of the buffering capacity of the cell surface (6, 9, 18). The cytoplasmic buffering capacity measurements reported here are not markedly different from data available from other studies of gram-positive bacteria (1, 9, 18) and of *Thiobacillus acidophilus* (23). Krulwich et al. (6) found significant differences between $B_t$ values of *E. coli* cells permeabilized with Triton X-100 and those permeabilized with n-butanol. The $B_t$ values of *S. marcescens* ATCC 274 in our study were comparable to the values found by Krulwich et al. with *E. coli* cells permeabilized with n-butanol over the $\text{pH}$ range studied. The pigmented strain *S. marcescens* ATCC 274 had higher $B_t$ values.

Passive $\text{H}^+$ conductance as well as the buffering capacity of these strains of *S. marcescens* was sensitive to the proton concentration at the external surface over the $\text{pH}$ range studied (Fig. 2). It is clear that the smooth curves that described the behavior of $C^{\text{H}_2}_m$ were different for the two strains and that strain ATCC 274 had $C^{\text{H}_2}_m$ values threefold higher than those of strain GP. The $C^{\text{H}_2}_m$ values of both strains of *S. marcescens* above $\text{pH}$ 5 were higher than those reported for gram-positive species (5, 6, 18), but there are no reports of membrane conductance to $\text{H}^+$ of aerobic suspensions of gram-negative bacteria that could be compared with our results.

The experiments described in this paper show that the buffering capacity and membrane conductance to protons of strain GP, a spontaneous mutant of ATCC 274 unable to produce prodigiosin, are lower than those of the wild-type strain. The pigment is synthesized in a bifurcated pathway in which monopyrrole (2-methyl-3-n-amyl-pyrrole [MAP]) and bipyrrrole (4-methoxy-2,2'-bipyrrole-5-carboxaldehyde [MBC]) moieties are produced separately and then combined to form prodigiosin. This condensation reaction is temperature sensitive (13, 22). Strain GP is able to produce MAP but not MBC. We had incubated both strains at 37.5°C to prevent the production of prodigiosin by strain ATCC 274. Thus, the difference between strains ATCC 274 and GP under the growth conditions described above was the production of MBC, a precursor of a secondary metabolite. Our results suggest that MBC and/or its precursors increase external and cytoplasmic buffering capacities and membrane conductance to $\text{H}^+$ in the $\text{pH}$ range studied.
In this article, we have proposed an alternative approach to study both the buffering capacity and the passive proton conductance of gram-negative cells based on the method described by Maloney (9). It will be of interest to study these parameters for other bacterial species because, as we have shown in our study, they may reflect differences in bacterial metabolism.

REFERENCES