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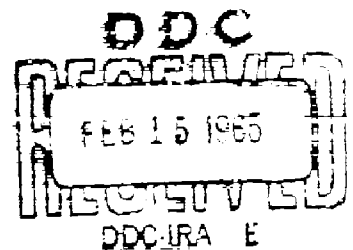
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OF NONPIGMENTED MUTANTS
IN PASTEURELLA PESTIS CULTURES

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SELECTIVE SURVIVAL OF NONPIGMENTED MUTANTS IN
PASTEURELLA PESTIS CULTURES

Dorothy M. Cavanaugh

Michael J. Surgalla

Medical Bacteriology Division
DIRECTOR OF BIOLOGICAL RESEARCH

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ABSTRACT

Differential death rates that occur under certain conditions in cultures of Pasteurella pestis result in dramatic population shifts.

Comparable growth and death rates were observed for pigmented inocula and their nonpigmented variants in a casein digest (NZ-Amine, Sheffield, Type A) medium containing added xylose and shaken at 26 C. A change of energy source from xylose to sodium gluconate had little effect on growth, but, in the death phase, greatly accelerated the death of pigmented cells while decreasing the death rate of nonpigmented mutants. These cultures in the death phase at 48 hours were often predominantly nonpigmented.

In gluconate cultures, the death rate of pigmented and nonpigmented populations could be returned toward normal (comparable) rates by substituting Na_2HPO_4 for the K_2HPO_4 (0.025 M) added during preparation of the medium. In gluconate- K_2HPO_4 cultures, the viability of both populations was prolonged by the addition of 0.1 M CaCl_2 or, to a lesser extent, of 0.2 M NaCl to the medium.

I. INTRODUCTION

One of the bacterial properties that has impressed plague investigators is the high rate of mutability of Pasteurella pestis to avirulent forms. The two most common avirulent forms are typified by the two widely used live vaccine strains, EV 76 and Tjiwidej, developed by Girard and Robic, and by Otten, respectively.¹ It is now known that the Girard and Robic strain has lost a property referred to as "pigmentation," but the Otten strain has lost a different property that includes ability to produce virulence (VW) antigens as well as a nutritional deficiency that is referred to as a "calcium requirement." Loss of virulence that is associated with loss of these two "virulence factors" is depicted diagrammatically in Figure 1.

These two common avirulent types have been seen and recognized by scientists working with plague for many years as differing from each other. However it was only in the last decade in Dr. Burrows² laboratory that all virulent strains were found to produce the VW antigens and to form black colonies on hemin agar. A temperature-dependent nutritional deficiency that is associated with the VW antigens was described in our laboratory,³ and this was concluded to be the same as that described independently by Higuchi et al.⁴ as being satisfied by calcium, strontium, or zinc. The ability to make VW antigens is lost at a rate of about 1 in 10,000 cell divisions.⁵ This seems to be true even if the pigmentation property is lost first. The rate of loss of pigmentation has not been determined but it appears to be at least as high as for VW, and is much the same in VW strains.

Loss of virulence associated with loss of virulence antigens has received much attention, and recent publications are concerned with an apparent alternative of VW antigen production or growth⁶ and ability of pyrimidine precursors to eliminate the growth advantage of avirulent mutants.⁷ This paper is concerned with cultural conditions that allow nonpigmented mutants to predominate in an aging culture by selective survival during the death phase.

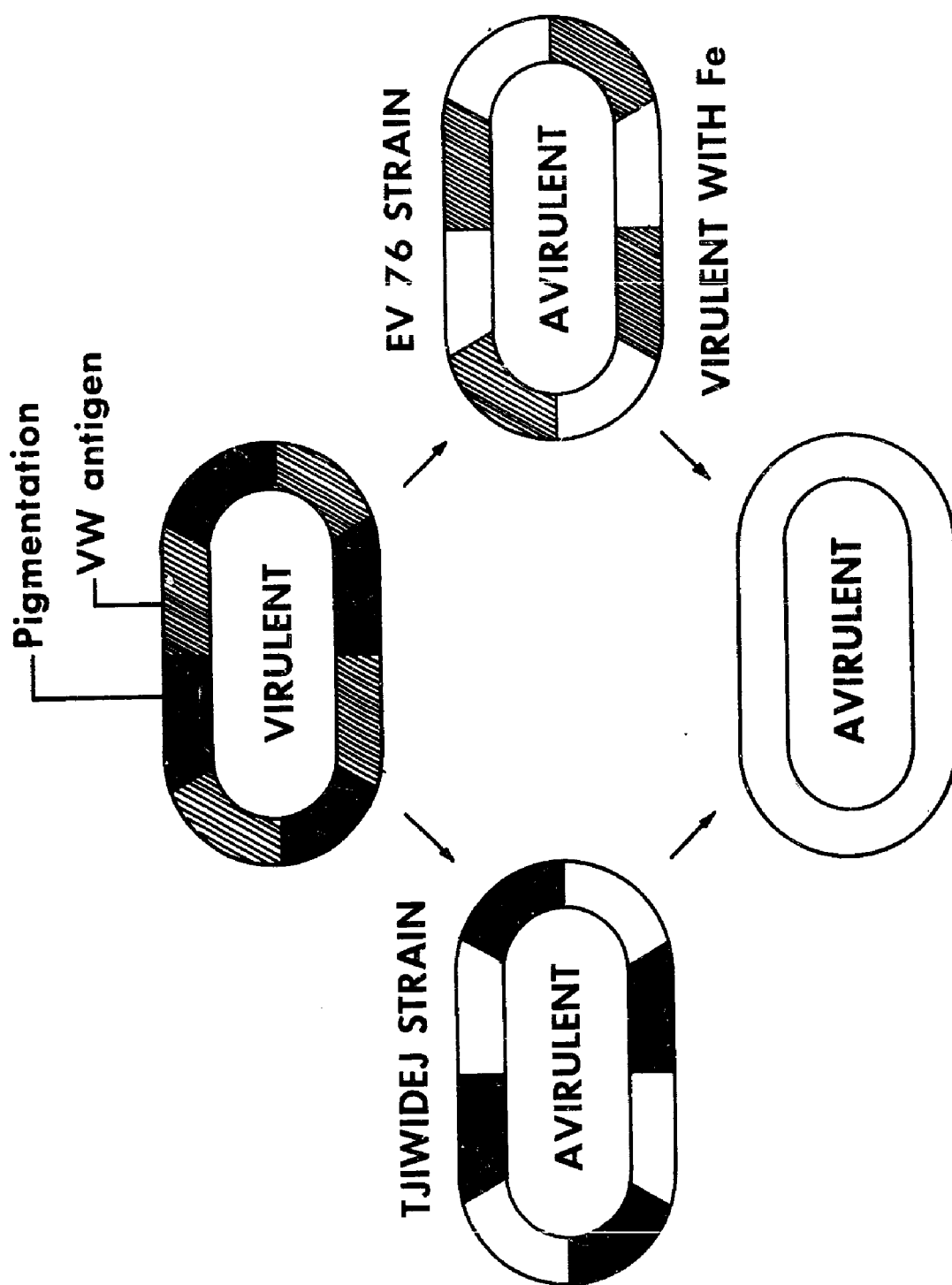


Figure 1. Diagrammatic Representation of Loss of Virulence Associated with Pigmentation and VW Antigen Virulence Factors.

II. MATERIAL AND METHODS

The virulent strain Alexander was used in the work reported here. A nonpigmented calcium-requiring type, as well as pigmented and non-pigmented avirulent types, were also tested.

Blood agar base (Difco) supplemented with 0.04% Na_2SO_3 and 0.1% glucose, with the pH adjusted to 7.2 to 7.4, was used for stock slants and total viable assay medium.

Magnesium oxalate medium (Mgox) of Higuchi and Smith⁵ was used to detect the VW type of avirulent cells in the virulent population and hemin agar of Jackson and Burrows⁸ was used to detect the nonpigmented mutants.

The basic liquid growth medium was NZ-Amine, Sheffield, Type A. The final medium included citric acid 0.01 M, sodium gluconate 0.01 M, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0025 M, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001 M, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.0001 M, and K_2HPO_4 0.025 M. It was adjusted to pH 7.2. Just before use, 0.0025 M sodium thiosulfate and a 1% energy source were added aseptically. This basic medium was altered as required in the different experiments.

Sampling was done with a syringe and 25-gauge needle in order to disperse as many clumps as possible. Dilutions were made in potassium phosphate buffer 0.06 M or in heart infusion broth (Difco).

All cultures were incubated at 26 C on a reciprocal shaker. The inoculum was grown for 16 hours with no added energy source, then added as a 5% inoculum to fresh flasks. The pH was measured before and during growth with a Beckman Model H2 pH meter.

The hemin plates were incubated at 26 C for 4 days, the blood agar base plates at 26 C for 48 hours, and the Mgox plates at 37 C for 48 hours.

III. RESULTS

In broth containing xylose as an additional energy source, the virulent inoculum, the nonpigmented mutants present, and the VW^- mutants all multiply and die at comparable rates (Figure 2). When sodium gluconate is substituted for xylose, the virulent cells die faster than nonpigmented mutants and the latter predominate after 48 hours.

In the gluconate broth, substitution of Na_2HPO_4 for the K_2HPO_4 in the medium eliminates the selective survival of nonpigmented mutants by causing faster death of these cells while slowing the death of the pigmented cells (Figure 3).

A similar differential response to increasing the Na and reducing K, resulting in slower death of pigmented and faster death of nonpigmented cells, was seen in VW^- cultures. Also, the unfavorable effects on nonpigmented populations were observed in pure cultures of nonpigmented cells isolated from either VW^+ or VW^- pigmented strains.

Additional information indicates that high pH favors survival of nonpigmented and hastens death of pigmented cells, and neutralization of the culture with HCl at 24 hours retards death of both types. The pH per se accounts for only part of the effect; KOH is far more detrimental than NaOH to pigmented cells. In gluconate- K_2HPO_4 cultures the viability of both populations is prolonged by the addition of either 0.1 M $CaCl_2$ or, to a lesser extent, 0.2 M NaCl to the medium.

Nothing is known of the mechanisms that cause pigmented and nonpigmented cells to give survival responses in opposite directions to the conditions described. Since possession of the pigmentation property by the bacterium is correlated with high mortality in the host, study of this property will continue.

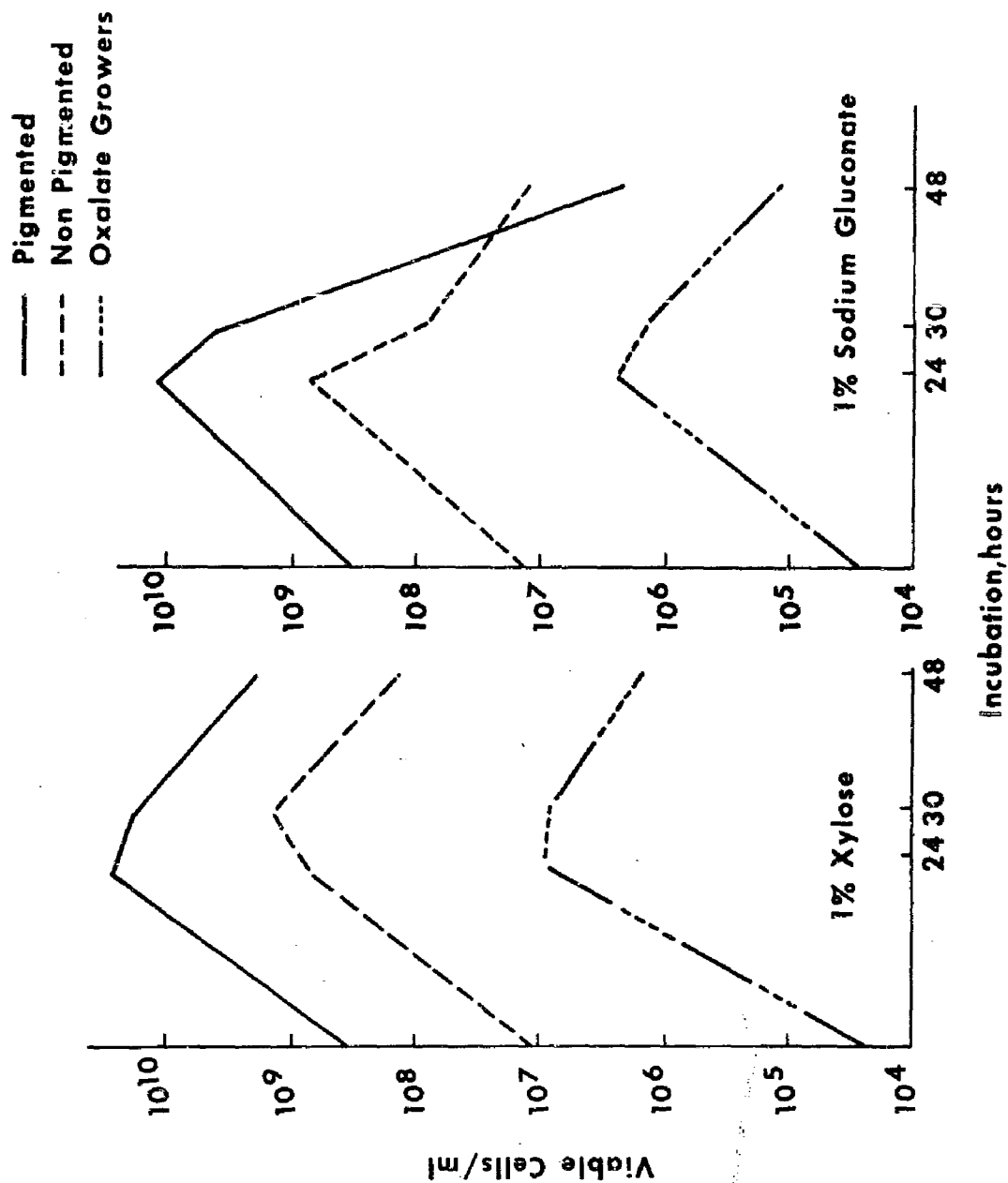


Figure 2. Comparison of Growth of Cell Types in NZ-Amine Medium with Xylose and Sodium Gluconate as Energy Sources.

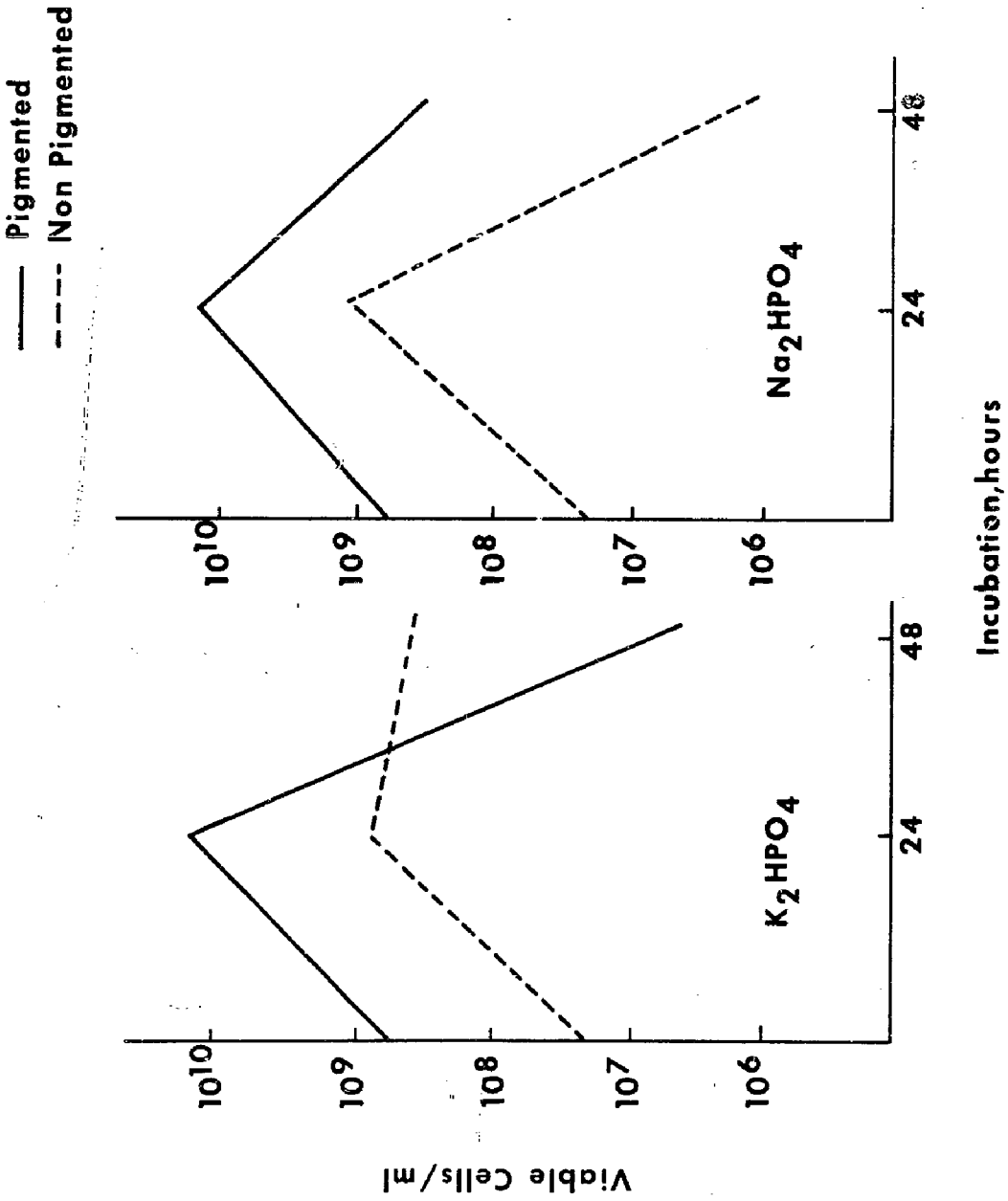


Figure 3. Comparison of Cell Types in NZ-Amine-Gluconate Medium with K_2HPO_4 or Na_2HPO_4 .

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