Acetylene Reduction by *Klebsiella pneumoniae* in Air Related to Colony Dimorphism on Low Fixed Nitrogen

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INTRODUCTION

Until recently it was believed that *Klebsiella pneumoniae* fixed nitrogen only under anaerobic conditions (see Postgate, 1971). However, Klucus (1972) showed that a species of *Klebsiella* produced active nitrogenase in the presence of O₂ in a chemostat culture provided that the medium contained yeast extract (1 g/l) as a fixed N source and the dissolved oxygen tension was at 10 mmHg or below. Klucus (1972) suggested that the tolerance of nitrogenase activity to O₂ arose because of a high respiratory rate at low dissolved oxygen tension (Harrison & Pirt, 1967; Harrison & Loveless, 1971) which could maintain the local oxygen tension at a very low level, although he did not measure respiratory rates. The obligate aerobe *Derxia gummosa* is particularly sensitive to O₂ when fixing N₂ (Hill & Postgate, 1969; Hill, 1971) and shows colony dimorphism on N-free media in air (Jensen, Petersen, De & Bhattacharya, 1960). Only the 'massive' colonies which develop among many 'thin' whitish colonies fix nitrogen (Jensen *et al.* 1960) or reduce acetylene (Hill, 1971). The numbers of colonies developing into the 'massive' type could be increased by either adding fixed nitrogen (Tchan & Jensen, 1963) or lowering the atmospheric oxygen concentration (Hill, 1971). Colony dimorphism probably arises in air because nitrogen fixation and subsequent growth to the 'massive' colony type takes place only where the local oxygen tension can become depressed (Hill, 1971). A methane-oxidizing bacterium also showed dimorphism during growth on a N-free medium in air (De Bont & Mulder, 1974). The present work demonstrates that *K. pneumoniae*, although probably more sensitive to O₂ when fixing N₂ than *D. gummosa*, also shows colony dimorphism associated with N₂ fixation when incubated in air if the medium is supplemented with low levels of fixed nitrogen.

METHODS

Organism. *Klebsiella pneumoniae* M5a1, a gift from Professor P. W. Wilson, was maintained at 20 °C in air on 2% (w/v) nutrient agar slopes and subcultured monthly.

Media. The N-free medium contained per litre: 15 g glucose, 12.06 g K₂HPO₄, 3.4 g KH₂PO₄, 26 mg CaCl₂·2H₂O, 30 mg MgSO₄, 0.3 mg MnSO₄, 36 mg ferric citrate, 7.6 mg Na₂MoO₄·2H₂O, 10 µg *p*-aminobenzoic acid, 5 µg biotin, 15 g agar (Bacto-agar, Difco), and, when required, from 2 mg to 2 g vitamin-free Casamino acids (Difco). The two vitamins are not in fact required by *K. pneumoniae*.

Incubation of plates under various atmospheric oxygen concentrations. Cultures inoculated on to solid media were incubated at 30 °C under atmospheric oxygen concentrations below 0.2 atm in nylon bags as described by Hill (1973).

Acetylene reduction. A portion (approximately 2 cm²) bearing colonies was cut from cultures grown on solid media in Petri dishes and placed in a serum bottle (7.5 ml). The bottle

Fig. 1. Colony morphology of *K. pneumoniae* on the medium containing 200 μg Casamino acids/ml after 8 days incubation at 30 °C in (a) air, (b) 0.25 atm air + 0.74 atm N_2 + 0.01 atm CO_2 or (c) 0.99 atm N_2 + 0.01 atm CO_2 . A progressively greater proportion of the colonies developed into the large yellowish-brown type than into the small white type when the atmospheric $p\text{O}_2$ was lowered.

was gassed with argon and capped with a Suba-Seal closure (William Freeman Ltd, Barnsley, Yorkshire). Oxygen was injected to the required atmospheric $p\text{O}_2$, followed by 0.8 ml C_2H_2 . After each injection the excess gas pressure was released. The bottles were incubated at 30 °C without shaking and gas samples (0.5 ml), removed for analyses for ethylene by gas chromatography, were replaced by 0.5 ml of the initial gas mixture of Ar or Ar + O_2 . Gas samples for analysis were injected into a Pye 104 chromatograph which was fitted with a flame ionization detector and a 45 cm column (1 mm internal diameter) filled with either Porapak R or Porapak N (mesh 100 to 120), and maintained at 37 °C with N_2 as carrier gas flowing at 7 ml/min. Ethylene peak heights were recorded and compared with standards.

RESULTS

Nitrogen-fixing cultures of *K. pneumoniae* were grown in liquid N-free medium either anaerobically in batch culture (medium contained 1%, w/v, glucose) or in an O_2 -limited chemostat culture (medium contained 0.5% glucose) at 30 °C, and organisms were inoculated on to the solid N-free medium containing 0, 20 μg , 200 μg , or 2 mg Casamino acids/ml. After 7 days in air at 30 °C, only very small white colonies were present with 0 or 20 μg Casamino acids/ml; these developed into large colonies when the plates were transferred to anaerobic conditions. With 2 mg Casamino acids/ml in air, all colonies were large and brown. These colonies reduced C_2H_2 when tested anaerobically or under 0.2 atm O_2 . With 200 μg Casamino acids/ml in air, most of the colonies were small and white but a few, in the sparsely inoculated areas, were large and yellowish brown (see Fig. 1a). Approximately similar areas of each colony type, grown for 8 days, were tested for C_2H_2 reduction. Blocks carrying the large, yellowish-brown colonies produced 62 to 129 nmol ethylene in 210 min when tested anaerobically, in 0.05 or 0.2 atm O_2 . Those with the small white colonies did not produce ethylene. Ethylene production by the large yellowish-brown colonies was progressive and was not observed in the absence of C_2H_2 . When the two colony types were separately inoculated on to the medium containing 200 μg Casamino acids/ml and incubated in air both subcultures showed a similar distribution of colony type. A progressively greater proportion of the large to the small colonies developed when cultures on this medium were incubated for 8 days in air, or in air diluted with different amounts of N_2 + CO_2 (see Fig. 1).

DISCUSSION

The two colony types were not genetically different since colony dimorphism was observed when either colony type was used as parent, the relative numbers of either type could be altered by changing the amount of fixed nitrogen or by altering the atmospheric pO_2 , and the small colonies developed into the large colonies when the atmospheric pO_2 was lowered. With 200 μg Casamino acids/ml, colony dimorphism in air was similar to that of *D. gummosa* (Jensen *et al.* 1960; Hill, 1971). With both *D. gummosa* and *K. pneumoniae*, large N_2 -fixing colonies probably develop predominantly in the sparsely inoculated areas, because sufficient fixed nitrogen diffuses from surrounding sterile areas to allow growth and accompanying respiratory activity to lower the local oxygen tension. Only then can N_2 fixation, and hence further growth, occur. In *D. gummosa* 'massive' colonies developed occasionally in densely inoculated areas where, it was suggested, competition for O_2 resulted in a low local oxygen tension (Hill, 1971). Similarly, a large colony of *K. pneumoniae* sometimes developed on the edge of a densely inoculated area (see Fig. 1a); this area reduced C_2H_2 . *Klebsiella pneumoniae* formed only small colonies in air on the N-free medium whereas *D. gummosa* under comparable conditions formed occasional 'massive' colonies, indicating that *K. pneumoniae* is more oxygen-sensitive when fixing N_2 than *D. gummosa*.

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