Abolition of Swarming of *Proteus* by *p*-Nitrophenyl Glycerin: Application to Blood Agar Media

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Comparative plate counts were made of *Staphylococcus aureus* and *Streptococcus pyogenes* growing on blood agar supplemented with individual chemicals to abolish the swarming of *Proteus*. *B*-phenylethanol, sodium azide, and *p*-nitrophenyl glycerin (PNPG) were used as anti-swarm agents. Each anti-swarm agent effectively abolished swarming for 24 h, but azide failed to control swarming for longer periods of incubation. In addition, azide displayed growth inhibition towards the staphylococci and streptococci resulting in no hemolysis and reduced viable cell numbers with the streptococci. Phenylethanol showed reduced viable cell numbers with the streptococci and unreliable hemolytic reactions. At 0.1 to 0.3 mM, PNPG proved to be a superior anti-swarm agent in that it showed no growth inhibition and allowed normal hemolysis, but abolished swarming for extended periods of time. When laboratory strains of *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Vibrio cholerae* were screened on a blood agar medium containing 0.1 mm PNPG, they displayed similar growth and hemolytic characteristics to the identical medium without PNPG.

A number of methods have been reported to prevent *Proteus* species from swarming over the surface of a solid culture medium, and thereby improve the methods of isolating clinically important bacteria (2; for a review with emphasis on the use of detergents see Lominski and Lendrum, reference 3). The addition of certain chemicals to culture media reportedly solved the swarming problem of *Proteus*; however, in practice all of these widely used agents have some drawbacks. The addition of sodium azide to blood agar media was reported to abolish the swarming of *Proteus* without affecting the isolation of clinically important staphylococci and streptococci (4), but blood agar media containing azide are not widely used in the clinical laboratory because azide turns out to be a poor anti-swarm agent and, as will be shown in this paper, it does show growth inhibition of certain streptococci. *B*-Phenylethanol is perhaps the most widely used anti-swarm agent in media for the isolation of gram-positive cocci; however, when it is used in a blood agar medium, hemolytic reactions cannot be reliably determined (1). Most laboratories use phenylethanol as an anti-swarm agent in spite of this obvious drawback. The new anti-swarm agent *p*-nitrophenyl glycerin (PNPG) seems to have a rather specific mode of action upon swarming without affecting motility or growth (5), thus potentially this agent may be superior to azide or phenylethanol in blood agar media.

This work is designed to evaluate azide, phenylethanol, and PNPG as anti-swarm agents in blood agar media used for the isolation of gram-positive cocci. This paper was presented in part at the 71st Annual Meeting of the American Society for Microbiology in Minneapolis, Minn., 2–7 May 1971.

MATERIALS AND METHODS

Organisms. The cultures of *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were obtained from the stock culture collection of the Department of Bacteriology, Iowa State University. Cultures of *Listeria monocytogenes* and *Vibrio cholerae* were obtained from the Veterinary Diagnostic Laboratory, Iowa State University. *Staphylococcus aureus* was isolated from a student in the General Bacteriology Course at Iowa State University and used in this study because it was strongly hemolytic and possessed strong pigmentation on blood agar. The
strain of S. pyogenes used in this work was also selected for strong beta-hemolysis. The strain of P. mirabilis was used in other studies of swarming performed in this laboratory (5).

**Culture media.** Blood agar plates were prepared from either Trypticase blood agar base (Difco), azide blood agar base (Difco), or phenethyl alcohol agar (BBL). To prepare a blood agar medium each base medium was supplemented with 5% sheep blood. In the evaluation of PNPG as an anti-swarm agent, Trypticase blood agar base was used. All petri plates prepared from the solid culture media were prepared overnight at 35°C to remove water droplets from the agar surface and also to check for contamination. Broth cultures were grown in Trypticase soy broth (Difco) at 35°C.

**Screening of hemolytic reactions and growth on PNPG blood agar.** Duplicate streak plates were prepared on blood agar with and without the addition of 0.1 mM PNPG. A variety of organisms were tested in this manner by directly comparing colony size, relative abundance of growth, and hemolytic reactions on the plates with and without PNPG.

**Comparative plate counts.** Overnight broth cultures were serially diluted in sterile distilled water, and 0.1 ml from each dilution was placed onto the surface of triplicate plates of Trypticase blood agar base, Trypticase blood agar base with 0.1 mM PNPG, phenethyl alcohol agar, and azide blood agar base, all supplemented with 5% sheep blood. The organisms were distributed over the surface of each plate with a sterile L-shaped glass rod. All plates were incubated for 24 h at 35°C, the colonies were counted, and the hemolytic reactions were observed. The plates were reincubated for an additional 24 h and again examined. Several plates of each medium were inoculated in the center with an undiluted broth culture of P. mirabilis to test the anti-swarm properties of each medium. These plates were incubated as the others and examined at 24 and 48 h for the presence of swarming.

**Chemicals.** PNPG was obtained from Sigma Chemical Co. and Regis Chemical Co. It was added to the basal medium and sterilized with the medium in an autoclave.

**RESULTS**

When cultures of S. aureus, S. pyogenes, S. pneumoniae, K. pneumoniae, P. aeruginosa, L. monocytogenes, and V. cholerae were screened by the streak plate technique, all of the organisms showed comparable amounts of growth, similar colonial morphology, and identical hemolytic activity on blood agar with and without 0.1 mM PNPG. Primarily because of the strong hemolytic reaction, S. aureus and S. pyogenes were chosen for further study with the different anti-swarm agents.

Table 1 shows the results of a comparative plate count study with S. aureus with the three anti-swarm media and unsupplemented Trypticase blood agar medium as control. No significant differences in viable cell numbers are seen with any of the test media, but the azide-supplemented medium shows partial growth inhibition for a 48-h period, as indicated by the pinpoint colony size on this medium. Proteus failed to swarm on any of the supplemented media for 24 h, but after 48 h, swarming was seen on the azide-supplemented medium.

The positive hemolytic reaction seen on the phenylethanol blood agar cannot be considered a reliable indication of hemolysis. This was probably due to lysis of red blood cells after interaction of phenylethanol (a detergent) with

| Table 1. Comparative plate counts of Staphylococcus aureus |
|-----------------------------------------------------------|
| Medium* | Colonies per plate at 10⁻¹ dilution | Colony morphology | Hemolysis | P. mirabilis control (24h) |
|---------|-----------------------------------|-------------------|----------|--------------------------|
|         | No. | Mean |                  |          | Growth Swarming          |
| Blood agar base | 127 | 121 | 109 | Normal | + |
| Blood agar base with | 108 | 118 | 131 | Normal | + |
| 0.1 mM PNPG |                  |                  |          | Growth No swarming       |
| Phenylethanol agar | 110 | 122 | 145 | Normal | + |
| Azide blood agar base | 137 | 120 | 117 | Pinpoint | - | Poor growth No swarming

*All media contain 5% sheep blood.*
excretion products surrounding the colonies.

A significant result of this test was that after 24 h all media containing an anti-swarm agent were effectively controlling swarming, but the PNPG-supplemented medium was the only one that permitted normal growth and colony formation of Proteus, the others all either severely inhibited or abolished growth of Proteus. This experiment was repeated using a level of 0.2 mM PNPG with identical results.

An identical comparative plate count experiment was performed with S. pyogenes as the indicator organism. The results of this experiment are shown in Table 2. With S. pyogenes, marked differences in viability of the organism on the different anti-swarm media are seen. Only unsupplemented and blood agar supplemented with PNPG permitted normal growth and colonial morphology. Neither azide-supplemented medium nor phenylethanol blood agar demonstrated cell viability comparable to the unsupplemented blood agar medium, and neither medium produced normal colonial morphology or hemolytic reaction in 48 h. In contrast, the PNPG blood agar medium allowed normal growth, colonial morphology and hemolysis in 24 h. The plates of anti-swarm medium inoculated with Proteus all controlled swarming for 24 h, but swarming was observed on the azide medium at 48 h. This experiment was also repeated using a higher level of PNPG in the blood agar medium (0.2 mM). The results were identical to those reported for 0.1 mM PNPG.

**DISCUSSION**

It is apparent from these results that PNPG represents a superior anti-swarm agent under the conditions of these experiments. The azide-supplemented medium showed growth inhibition of both the Staphylococcus and the Streptococcus used in these studies, and as a result no hemolysis could be seen. In addition, azide-supplemented medium failed to control the swarming of Proteus for more than 24 h. The phenylethanol-supplemented blood agar did an excellent job of preventing swarming of Proteus, apparently by acting as a growth inhibitor for this species, but the anti-swarm agent reduced the viability of the streptococci and is unsuitable if hemolytic reactions are to be determined. The PNPG-supplemented medium showed none of these drawbacks, and at the same time controlled swarming for at least 48 h. In addition, only the PNPG medium permitted Proteus to appear on the plate with normal growth and colonial morphology. This may be of great value to permit the clinical laboratory to assess the relative amounts of Proteus present in a given sample and at the same time permit the isolation of gram-positive cocci.

The screening tests indicated that a level of 0.1 mM PNPG did not show any detectable growth inhibition or adverse effects on colony morphology or hemolytic reaction on any of the organisms tested with the streak plate technique. No other levels of PNPG were tried, nor were comparative plate counts performed using these other organisms. These results would suggest that workers who encountered a problem with Proteus when attempting to isolate any of these other organisms should evaluate a PNPG-supplemented medium for their specific problem.

A question can be raised about the particular

| Medium* | Colonies per plate at 10^-3 dilution | Colony morphology | Hemolysis | P. mirabilis control (24 h) |
|---------|--------------------------------------|-------------------|-----------|----------------------------|
|         | No. | Mean |                      |           |                           |
| Blood agar base | 76  | 79   | 107                  | Normal    | Growth                     |
| Blood agar base with 0.1 mM PNPG | 87  | 74   | 93                   | Normal    | Growth                     |
| Phenylethanol agar | 22  | 16   | 10                   | Small     | Poor growth                |
| Azide blood agar base | No visible colonies | –        | –                    | No growth                |

*All media contain 5% sheep blood.*
strain of *P. mirabilis*, *S. aureus*, and *S. pyogenes* used in the comparative plate count experiments. These organisms are relatively far removed from a recent clinical infection or isolation, and they may no longer be representative of organisms freshly isolated in the clinical laboratory. I examined recent clinical isolates of *P. mirabilis*, and most are effectively controlled by 0.1 mM PNPG. However, in the case of two isolates, a level of 0.3 mM PNPG was required to stop swarming for 48 h in a Trypticase blood agar base medium. Because a number of different culture media are used as a base for blood agar, it is impossible to predict the optimal level of PNPG for each medium. In general, a blood agar base medium that does not contain yeast extract Trypticase blood agar base, blood agar base, heart infusion agar (Difco), Trypticase soy agar, blood agar base (BBL), or tryptose blood agar base (GBI) will require between 0.1 and 0.3 mM PNPG to arrest swarming for at least 48 h under aerobic conditions. All of these plates must be predried before use if the PNPG is to be effective because this agent will abolish swarming without affecting motility or growth, and *Proteus* will spread over the surface of the medium by motility through the water droplets on the surface of a freshly poured plate (5). Predrying removes these water droplets and at the same time permits detection of contaminated plates. If the base medium is supplemented with yeast extract, somewhat higher levels of PNPG will be required to abolish swarming (5).

Fresh clinical isolates of *Staphylococcus* and *Streptococcus* may differ in their sensitivity to PNPG, and perhaps toxicity will be observed. However, none of the stock culture strains of these organisms showed adverse effects when exposed to a level of 0.1 and 0.2 mM PNPG. It remains the task of the clinical laboratory to evaluate a PNPG-supplemented blood agar with actual clinical specimens and to compare PNPG with media containing other anti-swarm agents. This is truly the environment in which an anti-swarm agent must be evaluated. These results indicate that such an evaluation is necessary and, since no adverse effects of PNPG have yet been observed, I'm extremely optimistic about the clinical use of this agent.

**LITERATURE CITED**

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