

Full Length Research Paper

Comparative biochemical and molecular evaluation of swarming of *Proteus* and effects of anti-swarm agents

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In addition to inadequate understanding of swarming motility and virulence of *Proteus*, there is paucity of information on the relative effectiveness of the various anti-swarm agents. The anti-swarming effects of urea, sodium dodecylsulphate (SDS) and trihydroxymethylglycine (Tris) on 40 clinical isolates of *Proteus* Spp. were comparatively investigated and plasmids associated with swarming were characterized. The three substances elicited a comparable concentration-dependent anti-swarming property at 0.25 – 1.25% on nutrient agar. Anti-swarm agents displayed heterogeneity in their ability to cause significant decreases in the expression of virulence factors. Swarm motility was further found to be strongly associated with the expression of virulence factors in these strains. Of the *Proteus* strains tested, 32 were found to harbour 1 – 4 plasmids of size ranging from 6.0 – 33.5 kb. Plasmid curing resulted in loss of swarming in 65.6% of these strains. In order to reduce the risk of infection with virulent *Proteus* strains, the laboratory use of urea and SDS is suggested.

Key words: *Proteus*, swarming, urea, SDS, Tris.

INTRODUCTION

Proteus species contribute greatly to neonatal mortality among Africans with urinary tract infections and are a leading cause of bacteruria associated pregnancy loss worldwide (Orrett, 1999; Cutner et al., 1992). In Nigeria, *Proteus* strains are frequently isolated from patients with kidney diseases, benign prostate hypertrophy and indwelling catheters and have assumed a leading role next to *Pseudomonas aeruginosa* as potential nosocomial pathogens in most Nigerian hospitals and a threat to public health (Kesah et al., 1992; Adeyemo, 1994).

Cases of re-current bacteruria are very common in developing countries in particular and this have been blamed on culture diagnostic inappropriateness caused by swarming motility of *Proteus* and related organisms (Williams and Schwartzhoff, 1978; Harshey, 1994). VanAsten and Gaastra (1999) reported difficulties in identifying *P. aeruginosa* and *Streptococcus G* in mixed cultures involving *Proteus*. This scenario may contribute to spread of multidrug resistance strains and treatment failures of polymicrobial infections. Efforts to annul the effects of swarming and enhance diagnosis through culturing in minimal media often fail to identify co-pathogens with *Proteus* that are fastidious or require extra nutrient supplements for growth (Reynolds and Penn, 1994).

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However, on solid media many substances have been found to demonstrate anti-swarm properties. They include urea (William, 1973), ethanol and sodium azide (Hernandez and Cavallo, 1999), barbitone and activated charcoal (Alwen and Smith, 1967). Recently, swarming refraining effect of urea was demonstrated on clinical isolates of *Proteus* from Lagos, and at 0.75 – 1-25%. Urea was found to refrain swarming and allow identification of *Klebsiella pneumoniae* and *Staphylococcus aureus* without compromising extended beta lactamases strain identification (Iwalokun et al., 2003). However, knowledge concerning whether urea influence the expression of virulence factors among these strains is not yet advanced. Elsewhere, multiple drug resistant and biofilm producing swarmed cells have been found to show sensitivity to sodium dodecyl sulphate (SDS) (Davies et al., 1998). The latter seem to support our observation that SDS also refrained swarming greatly in *P. aeruginosa* and *Proteus*. Trihydroxymethylglycine (Tris) is widely used in buffer systems involving microbial culture and enzymology, biotechnology and genetic engineering but there have not been clues to show whether this substance could inhibit translocation of bacteria on solid media *in vitro*. In developing countries, anti-swarm agents are ideally expected to be highly effective, readily available for routine use and confer reduction in the risk of transmission of pathogenic organisms.

Generally, the roles of plasmids in swarming motility have experimentally been demonstrated (Belas et al., 1998) but there is still insufficient information on plasmid profiles in local clinical isolates of *Proteus*. Such information could increase understanding on whether swarming-related genes are plasmid borne.

Hence the present study, which enrolled forty *Proteus* strains, was designed to compare and evaluate the biochemical effects of SDS, urea and Tris on total protein, carbohydrate content, cellular RNA and the expression of extracellular protease in *Proteus*. The involvement of plasmids in swarm motility in these isolates was also determined.

MATERIALS AND METHODS

Proteus strains

A panel of 40 newly isolated *Proteus* strains showing swarming motility on nutrient and broth agar plates were recruited for study. The isolates were obtained from hospital samples (urine, wound exudates, discharging ear specimen, vagina discharge and diarrhoeic stool) and identified in the Microbiology and Genetic Department of the Nigerian Institute for Medical Research, Lagos, Nigeria according to Cowan (1974)

Effects of Tris, urea and SDS on swarming

Cultures were grown to late exponential phase ($A_{625nm} = 0.7 - 0.9$) and 2 μ l of 10^8 cfu/ml of each *Proteus* strain was inoculated at the

center of grown in nutrient agar medium containing 0.3% meat extract, 1% peptone and 1.8% Eiken agar and supplemented with Tris, SDS or urea at 0.25 – 1.25% concentrations. Inoculated plates lacking Tris, SDS and urea were also prepared and used as positive controls. The plates were then incubated at 37°C for 24 h under aerobic conditions. The outer diameter of swarming zone from the point of inoculation was measured in millimeter (mm).

Cell-free protein determination

An overnight nutrient broth culture of each of the *Proteus* strains was centrifuged at 2000 x g for 10 min and the resulting supernatant was used for total protein determination according to Lowry et al. (1951). Bovine serum albumin (500 – 3500 μ g/ml) was used as a standard.

Effects of Tris, urea and SDS on Carbohydrate content

The extracellularly secreted carbohydrates in the *Proteus* strains were quantitated from culture supernatants using the anthrone method (Wharton and McCarty, 1972). The secreted carbohydrate was determined in the presence and absence of the anti-swarming agents.

Effects of Tris, urea and SDS on cellular RNA concentration

Cellular RNA content was estimated following cell lysis according to Jayaraman (1988). Cells were lysed by the addition of 400 μ l 4% SDS in Tris EDTA containing 0.4 N NaOH. 300 μ l of sodium acetate buffer (pH 5.2) was added five minutes later to stop lysis. Cell debris was removed by centrifugation at 3000 x g for 10 min. Yeast RNA (150 – 200 μ g/ml) was employed for calibration curve preparation.

Urease assay

One ml aliquot of *Proteus* culture supernatant was added to 1ml of 0.2 M phosphate buffer containing 3% urea and incubated for 15 min at 55°C. Reactions were terminated by quick transfer to ice and addition of 1 ml 0.66 N H₂SO₄. Protein was estimated according to Lowry et al. (1951) and the liberated ammonia was determined colorimetrically at 500 nm using Nessler's reagent (Jayaraman, 1988). Specific activity of urease was measured as moles of ammonia liberated/mg protein/min.

Protease assay

Extracellular protease in broth culture supernatants of the *Proteus* strains treated and untreated with the anti-swarming agents was assayed for using the protocol of Green et al. (1989). One unit of enzyme activity was defined as the proteolysis of 1 μ g of azocasein substrate per hour at pH 7.0 and 25°C.

Plasmid extraction

Plasmid DNAs from *Proteus* strains treated and untreated with anti-swarm agents were extracted using the alkaline lysis procedure of Takahashi and Nagano (1976). The extracted plasmids were electrophoresed on 0.8% ethidium bromide pre-stained agarose gel with tris-borate buffer (pH 8.6). Plasmid DNAs were electrophoresed in parallel with standard molecular weight markers (3.5 – 25.2 kb) to allow extrapolation of their sizes.

Plasmid curing

Proteus strains harbouring plasmids were cured of their plasmids according to Garriga et al. (1993). An overnight culture of each *Proteus* strain standardized to 1×10^8 cfu/ml with phosphate buffered saline (pH 7.2) was subcultured at 5×10^5 cfu/ml into 30 ml nutrient broth (pH 7.6) containing 1.25% ethidium bromide solution. Cultures were incubated for 72 h with continuous shaking (120 rpm) under aerobic condition. Cell pellets were then obtained by centrifugation (5000 rpm, 10 min, 4°C). Curing is indicated by loss of plasmids and confirmed by agarose gel electrophoresis described earlier. Swarming motility of cured *Proteus* strains was also investigated in nutrient agar as described previously.

Statistical analysis

Data were expressed as means and means \pm standard deviation. Regression analysis was used to predict associations and evaluate relationships between parameters. Generally, relationships or associations with $P < 0.05$ were considered significant and strong.

RESULTS

The effects of urea, Tris and SDS on swarm motility, extracellular protease and urease activity, cellular RNA and carbohydrate content among the 40 *Proteus* strains tested are presented in Figures 1 - 5. Swarming motility indicated by outer colony diameter of the strains on agar was observed to decrease significantly ($P < 0.05$) with increasing concentration of the anti-swarm agents (Figure 1). Concentration dependent reduction in urease activity was also observed with SDS and urea eliciting greater reduction effect on enzyme activity (Figure 2). Similar result was observed in the extracellular protease assay (Figure 3). The anti-swarm agents were also observed to elicit comparable reduction in cellular carbohydrate and RNA levels in the *Proteus* strains tested (Figures 4 and 5).

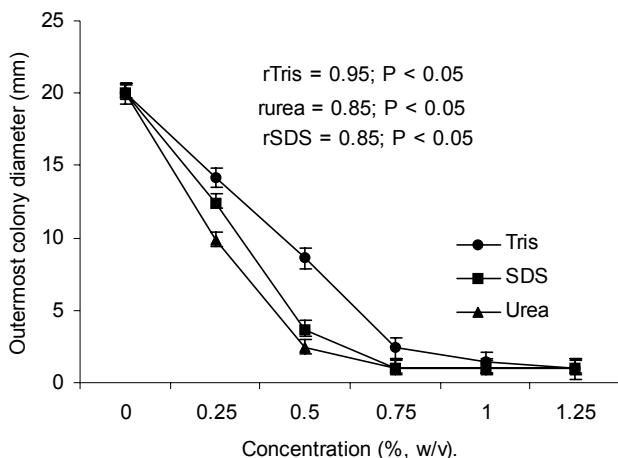


Figure 1. Swarming inhibitory property of SDS, Tris and urea among *Proteus* isolates from Lagos, Nigeria.

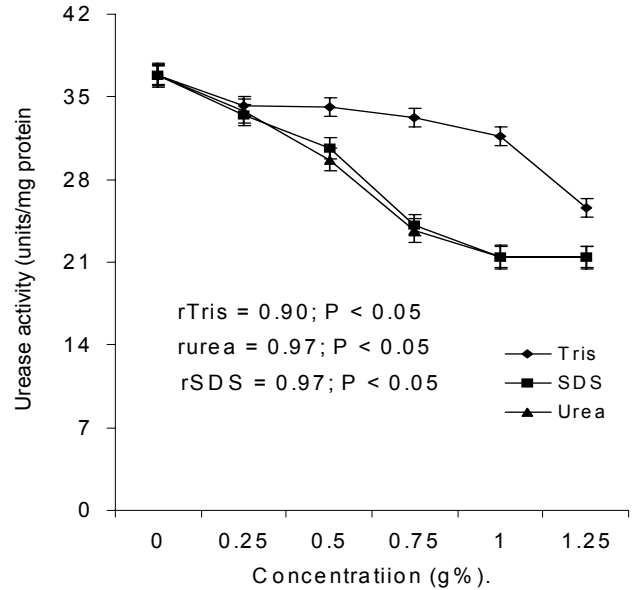


Figure 2. Effects of Tris, urea and SDS on urease activity among the *Proteus* strains.

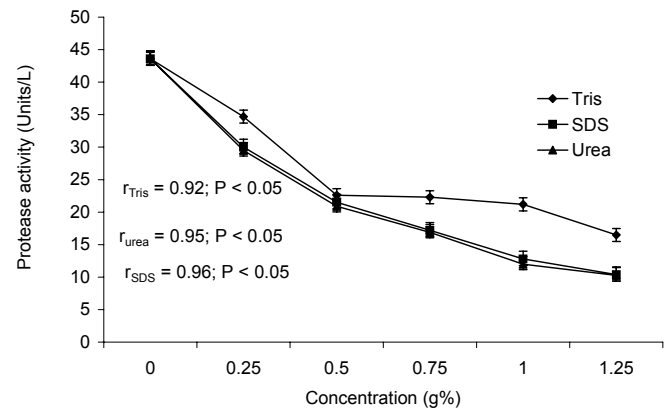


Figure 3. Effects of SDS, urea and Tris on extracellular protease activity among the *Proteus* strains.

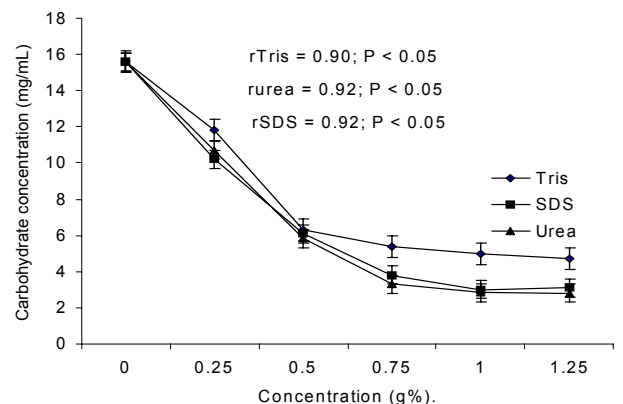


Figure 4. Effects of Tris, urea and SDS on carbohydrate expression among the *Proteus* strains.

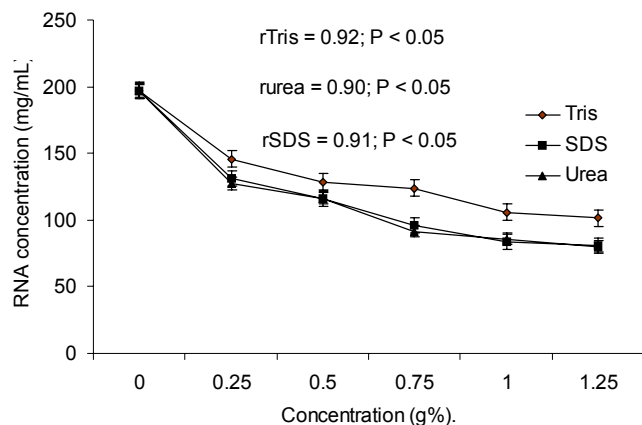


Figure 5. Effects of Tris, urea and SDS on cellular RNA level among the *Proteus* strains.

Unlike Tris, SDS and urea generally caused a sharp drop in cellular carbohydrate and RNA as well as extracellular urease and protease activity at 0.5 – 1.0 % concentrations (Figures 2 – 5). Furthermore, regression analysis for the assessment of the relationship between swarm motility inhibition and expression of virulence factors in the tested *Proteus* strains revealed strong association of refrained swarming with protease activity, urease activity, cellular RNA level and carbohydrate expression.

Plasmid analysis revealed that 8 strains were plasmidless, while the remaining strains harboured 1 – 4 plasmids of size ranging from 6.0 – 33.5 kilo base pairs. Treatment with the anti-swarming agents and curing with ethidium bromide were observed to result in plasmid loss (Table 1, Figure 6). Swarm motility was observed in 11 of 32 strains cured of their plasmids.

Table 1. Plasmid analysis of the *Proteus* isolates.

Number of <i>Proteus</i> isolates	No. of plasmids	Plasmid size (Kb)
8	0	-
15	1	6.0, 10.5, 12.5, 14.0
12	2	6.0, 10.5, 12.5, 14.0, 20, 33.5
4	3	6.0, 10.5, 28.0, 33.5
1	4	6.0, 10.5, 33.5

DISCUSSION

Swarming motility, a surface dependent coordinated multicellular behaviour has been established to be the basis for colonization and expression of virulence factors by pathogenic organisms including *Proteus*. This phenotype is a diagnostic constraint and a barrier to adequate anti-proteus measures in humans and animals

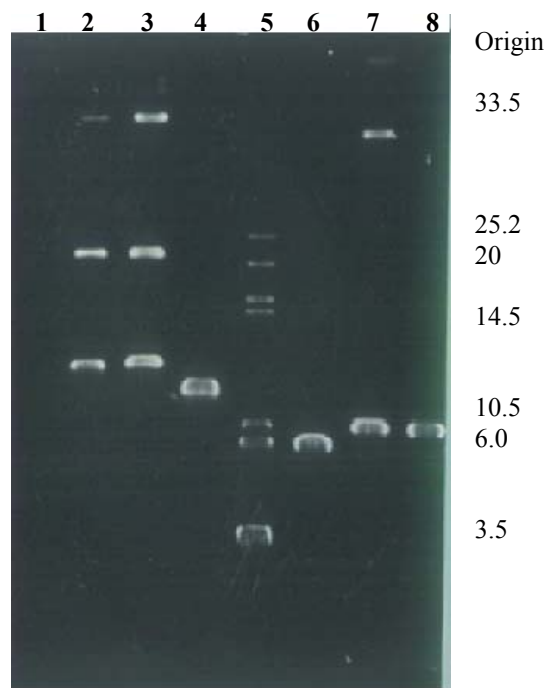


Figure 6. Plasmid profile analysis of *Proteus* strains with swarming refrained by Sodium dodecylsulphate, Tris and urea. Lane 1 = Negative control; Lanes 2 and 3 = untreated *Proteus* strains; Lane 4 = *Proteus* strains treated with SDS; Lane 5 = DNA markers (3.5 – 25.2 kb); Lane 6 = Plasmid cured *Proteus* strains; Lane 7 = *Proteus* strains treated with Tris; Lane 8 = *Proteus* strains treated with urea. Band sizes were measured in kilo base pairs (kb).

(Iwalokun et al., 2003; VanAsten and Gaastra, 1999). The present study has provided the molecular and biochemical consequences of the compared swarming refraining properties of urea, SDS and Tris.

The observed swarming refraining action of urea similar to that of SDS is in consonance with previous reports (Williams, 1973; Hernandez and Cavallo, 1999). The ability of SDS to inhibit swarming motility may not be unconnected with the demonstrative evidence of its biocidal effect on signal molecules involved in biofilm formation by *P. aeruginosa* (Davies et al., 1998). Furthermore, Campanac et al. (2002) recently demonstrated biocide cationic agents as suppressor of biofilm formation in pathogenic organisms. Meanwhile, previous studies among members of the *Enterobacteriaceae* have successfully demonstrated the coupling of swarming motility to biofilm formation with this relationship strongly established in *Yersinia enterocolitica* (Young et al., 1999).

To further improve understanding on the consequences of swarming motility inhibition on virulence, we investigated the effect of the anti-swarming agents on urease and protease activity, cellular RNA level and carbohydrate expression in the 40 *Proteus* strains tested. These substances were observed to cause marked

reduction in these parameters, contrary to the previously reported roles played by serine, methionine and glutamine (Iwalokun and Akinwunmi, 2002). The reduced cellular RNA may be a manifestation of gene repression mimicking the observation of Rasmussen et al. (2000) that brominated furanones, inhibit swarming genes transcription and the work of Liaw et al. (2003) in which inhibition of virulence gene expression was linked to mRNA degradation in *Proteus mirabilis*. The observed decline in protease and urease activity is supportive to the findings in which p-nitrophenylglycerin was used to inhibit swarming differentiation in *Proteus in vitro* (Liaw et al, 2000). Resistant isolates were subsequently found to display super – swarming phenotype (Liaw et al., 2001).

The concentration dependent mode of swarming motility inhibition demonstrated in this study further reveals the possible use of these substances at low concentrations to achieve distinct colony cultures from polymicrobial specimen. From diagnostic perspective, such concentrations may not alter antibiotic susceptibilities of the isolated organisms as recently observed in urea (Iwalokun et al., 2003).

The components lipopolysaccharide and capsular polysaccharide in cell matrix constitute the carbohydrate requirements for almost all swarming pathogens including *Proteus* spp. (Consterton, 1987; Consterton et al., 1999). Mutant *Myxococcus xanthus* strains lacking O-LPS gene expression also lacked the ability to swarm (Bowden and Kaplan, 1998), while glycogen synthesis and swarming motility deficiencies have been observed in *Escherichia coli* deficient in CsrA, a pleiotropic RNA - binding protein gene (Romeo et al., 1993; Wei et al., 2001). Gygi et al. (1995) also reported the involvement of a surface polysaccharide in swarming motility in *Proteus mirabilis*, similar to the spontaneous release of lipopolysaccharide by pathogenic *P. aeruginosa* (Cadieux et al., 1983). Our observed reduction in carbohydrate levels among the 40 treated *Proteus* strains seems to validate these findings.

Swarming motility, as a quorum sensing behavior, has been demonstrated in several signal transduction experiments to involve many distinct genes that promote intercellular communication (Fuqua et al., 2001), synthesis of biosurfactants (Eberl et al., 1999) and virulence factors (Giskov et al., 1997). These findings have subsequently been validated by the works of Belas et al. (1998) and Duffour et al. (1998) where transposons mutants were employed to establish swarming motility and virulence mediated gene expressions. The localization of swarming and virulence genes within transposons implies that these genetic determinants are mobilizable (Duffour et al., 1998). Plasmids are extrachromosomal genetic elements that have also been implicated in the spread of resistance and virulence across species of pathogens through mobilization (Gebre-Yohannes and Drasar, 1988; Hale et al., 1984). The persistence of swarming motility behaviour in some of the *Proteus* strains after curing strongly suggests likely

the involvement of chromosomal DNA in swarming. While the observed unique plasmids of size 6.0, 12.5, 20 and 33.5 kb further implies the involvement of plasmids in this motility behaviour in *Proteus*. Therefore, studies aimed at defining the roles, regulation of plasmids and chromosomes associated with swarming in *Proteus* and other related organisms may further provide an insight on the dynamics of swarming motility behaviour and gene expression.

Based on the data obtained from this study, it can be concluded that swarming motility in *Proteus* may be coupled to the expression of plasmid genes, and is inhibited by SDS, urea and Tris *in vitro*.

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