Superoxide involvement in the bactericidal effects of negative air ions on *Staphylococcus albus*

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The physical nature of small air ions is well established and it is recognised that they can produce a variety of biological effects[1]. However, in only a few instances have any underlying biochemical changes been detected[2–4]. Theoretically, one can consider the hydrated superoxide radical anion (O_2^-) $(H_2O)_n$ with $n \simeq 4$–8 as a likely candidate for a biologically active species of negative air ion[5]. The chemical and biological reactivity of superoxide is high[6] and includes a leading role in bacterial killing caused by radiation[7,8], in which superoxide dismutase (SOD), an enzyme that catalyses the reaction:

$$O_2^- + O_2^- + 2H \rightarrow H_2O_2 + O_2$$

protected markedly. Other studies have also demonstrated the bactericidal effect of O_2^- (refs 9–11). Inasmuch as the bactericidal action of small negative air ions has been repeatedly confirmed, we decided to test for the involvement of O_2^- in this phenomenon by evaluating the protective effect of SOD. Our results show strong O_2^- involvement in negative air ion bacterial kill.

Figure 1 illustrates the apparatus used in our experiments. To ensure that all negative ions went through the solution to the ground wire, we insulated the ground wire using heat shrink tubing and Teflon tubing to a point well below the solution's surface. A Modulion corona discharge type negative ion generator provided the ionising potential; to maintain a uniform current we adjusted the ionising potential using separate variable transformers on the a.c. lines of each generator.

Inoculated flasks, containing *Staphyloccus albus* at $\sim 10^6$ cells per ml in 40 ml of sterile 3.75 mM NaCl with 1.25 mM KPi, *p*H 7.8 buffer, were maintained at 22 ± 1 °C in a waterbath for the duration of the experiment. A vacuum pump with airflow regulator exhausted air from the flasks at a rate of approximately 500 ml per min. Aliquots of 1 ml were removed hourly, serially diluted, and then plated as previously described [12].

We isolated superoxide dismutase from bovine liver using the procedure of McCord and Fridovich[13]. This yielded pure enzyme of high specific activity (~3,500 U ml^{-1}). Catalase (Boehringer Mannheim) was extensively dialysed before use.

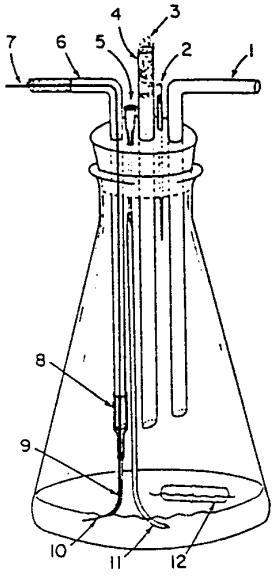

Fig. 1 1, Air exhaust tube. 2, Ion discharge needle. 3, Cotton plug. 4, Air inlet tube. 5, 18 gauge hypodermic needle. 6, Ground wire tube. 7, Ground wire. 8, Heat shrink tubing. 9, 1-mm Teflon insulating tube. 10, Bare platinum tip. 11, 1-mm Teflon sample tube. 12, Magnetic stirring rod. The apparatus was constructed in a 500 ml Erlenmeyer flask fitted with a no. 10 rubber stopper.

Enzymes were sterilised by passage through a Millipore filter immediately before addition to the bacterial inoculum.

Table 1 shows that the exposure of bacteria to negative air ions caused a substantial loss in viability in as short a period as 2 h, with a total loss of viability seen after 5 h of ion treatment. Control samples, incubated in identical conditions but with no negative ionisation, retained full viability after 5 h. Addition of 10 μg ml^{-1} SOD protected bacteria completely against negative ion killing while catalase or denatured SOD had no significant protective effect. The lack of effect of catalase in this system appears in marked contrast to that seen in most systems which involve O_2^- toxicity. For example, both O_2^- and H_2O_2 were required for lipid peroxidation or erythrocyte lysis[14] where their toxicity manifests through their interaction to produce hydroxyl radical and singlet oxygen. In the case of negative air ion bacterial kill the toxicity of O_2^- appears H_2O_2 independent. A possible mechanism might involve O_2^- acting as a nucleophile on the phospholipid bilayer, causing a de-esterification of fatty acids[15]. This could lead to an increase in surface charge and a weakening of the membrane, which under hypotonic conditions might lead to cell lysis and death.

The experiments reported here support the concept that the negative air ions responsible for bacterial death consist solely or in part of hydrated superoxide anions. Paradoxically, while negative air ions have a reputation as a rather beneficient species[1], superoxide radical anion apparently acts as one of the

Table 1 Bacterial kill by negative air ions (% viability)

Conditions	Incubation time (h) 0	1	2	3	4	5
No (−) ions (control)	100	123 ± 3.4	115 ± 22.5	112.5 ± 1.0	111.6 ± 22.3	105.6 ± 11.0
(−) Ions	100	116 ± 3.5	72.3 ± 9.9	29.2 ± 24.9	2.5 ± 2.3	0
(−) Ions +10 μg ml^{-1} SOD	100	129.0 ± 1.0	106.2 ± 18.8	134.0 ± 13.0	109.0 ± 25.1	104.8 ± 14.2
(−) Ions +10 μg ml^{-1} (denatured autoclaved SOD)	100	87.9 ± 7.1	70.0 ± 18.1	27.1 ± 1.1	5.5 ± 1.4	0
(−) Ions +10 μg ml^{-1} catalase	100	105.2 ± 23.8	56.7 ± 10.4	21.5 ± 4.4	11.9 ± 3.4	1.0 ± 1.0

Negative ion flux was maintained at approximately 5 μA in all experiments. ± Figures indicate the range between duplicate experiments.

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most toxic species produced in aerobic metabolism[6]. Perhaps the resolution of this paradox lies in the relative amount of O_2^- required for an effect. Negative air ions can cause physiological effects at extremely low levels, with clean country air having a negative ion concentration of only about 10^3 cm^{-3}, an amount which more closely parallels that of pheromones than of chemical reactants. In our system we have greatly amplified the flux of negative ions to a level which could produce, theoretically at least, 0.3 mmol O_2^- per h, which we channelled through a total of 40 ml of solution. Even so, *in vitro* systems testing for O_2^- reactivity have invoked a flux hundreds of times greater. Thus the effects of superoxide as a negative air ion although dependent on its chemical reactivity may be greatly amplified by a biological sensitivity in the hormonal range. Our tentative identification of $(O_2^-)(H_2O)_n$ as the negative air ions responsible for killing micro-organisms should provide a more solid base for investigating the effects and mechanisms of negative air ion effects in other biological systems.

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Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion

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We have previously regenerated tobacco plants from fused protoplasts isolated from two varieties of *Nicotiana tabacum*. The parent cells had distinct morphological nuclear and cytoplasmic markers, enabling us to recognise, among the whole regenerated plants, those with a single parental nucleus and a hybrid cytoplasm produced by the mixing of the two parental cytoplasms[1]. Genetic analysis of their progeny has confirmed that the phenotypes of these cytoplasmic hybrids (or cybrids) are stable and maternally inherited. Analysis with restriction endonuclease has shown that only one or the other parental chloroplast DNA is present in the progeny of the cybrids[2]. We report here, however, that the mitochondrial (mt) DNAs of cybrids are different from those of the parents and from the mixture of the two. The new DNAs result from mitochondrial recombination.

Cytoplasmic hybrids were obtained from fusion between protoplasts of *N. tabacum* var. Xanthi (Xf) which has normal fertile flowers and petiolated leaves, and *N. tabacum* var. Techne (Ts), which has abnormal flowers with cytoplasmic male sterility, producing no pollen. The Ts variety, obtained from the interspecific cross *N. debneyi* × *N. tabacum*, has the cytoplasm of the former and the nucleus of the latter. The resulting nucleocytoplasmic interaction is responsible for the male sterility. Ts has sessile leaves, and in our experimental system leaf shape is the nuclear marker and flower shape the cytoplasmic marker. Details of the parents and cybrids are shown in Table 1.

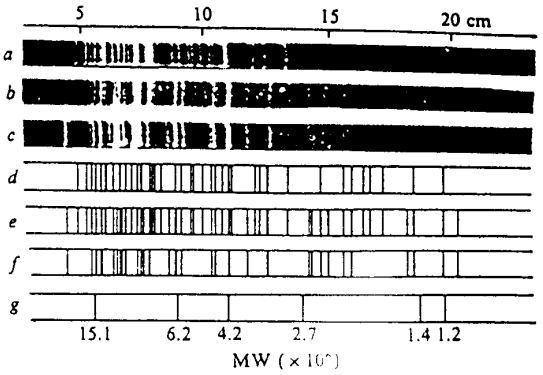

Fig. 1 Agarose slab gel electrophoresis of *Sal*I digests of mtDNAs from: *a*, Ts or *N. tabacum* var. Techne (*debneyi* cytoplasm); *c*, Xf or *N. tabacum* var. Xanthi (*tabacum* cytoplasm); *b*, mixture of Ts and Xf. These diagrams are schematically represented on *d*, (Ts); *e*, (Ts + Xf); *f*, (Xf). *Hind*III fragments of λ DNA (g) were used as standards for molecular weight determinations. Mitochondria were isolated as previously described[3] with the following modifications. Green leaves were used because etiolated material cannot be obtained with tobacco. DNase-treated organelles were purified by centrifugation on two layers of 30 and 60% sucrose. mtDNA was extracted as described for cp-DNA[4,7] and purified by linear CsCl–ethidium bromide gradient centrifugation for 2 d at 32,000 r.p.m. in a SW41 rotor. This gives cleaner mtDNA bands than does the two-step procedure used before. *Sal*I enzyme was prepared according to R. J. Roberts (unpublished). Experimental conditions for mtDNA digestion, agarose gel electrophoresis and UV fluorescence photography were as described[3,7].

Restriction endonuclease was used to analyse mtDNAs extracted from the progeny of nine cybrids (six Ti and three Xi) which represent all the variability of the new i phenotypes (see legend to Table 2). Mitochondria and mtDNAs were isolated as described in Fig. 1 legend.

Figure 1 shows the *Sal*I restriction patterns for mtDNA from the Ts (*a*) and Xf (*c*) parents showing 38 and 31 fluorescent bands, respectively. Of these, 20 appear to be common to *N. debneyi* and *N. tabacum* mtDNAs, 18 specific to Ts (*N. debneyi*) mtDNA and 11 specific to Xf (*N. tabacum*) mtDNA, as shown by comparison of Ts, Xf and Ts + Xf schematic diagrams (Fig. 1). Consequently, the diagram corresponding to a mixture of the two parent mtDNAs has 49 bands (Table 2). We found that the two varieties of *N. tabacum*, Xf and Tf (fertile Techne containing the *N. tabacum* cytoplasm), had identical mtDNAs. Figures 2 and 3 show the *Sal*I restriction patterns of mtDNAs

Table 1 Genotypes and phenotypes of parent and cybrid plants

Name		Genotypes: Nucleus	Genotypes: Cytoplasm	Phenotypes: Leaf	Phenotypes: Flower
Parents	Ts	*N. tabacum* var. Techne	*N. debneyi*	Sessile	Abnormal; male sterile type s
	Xf	*N. tabacum* var. Xanthi	*N. tabacum*	Petiolated	Normal; male fertile type f
Cybrids	Ti	*N. tabacum* var. Techne	hybrid *N. debneyi* × *N. tabacum*	Sessile	Intermediate between those of the parents
	Xi	*N. tabacum* var. Xanthi	hybrid *N. debneyi* × *N. tabacum*	Petiolated	

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