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# Nucleases of bacterial pathogens as virulence factors, therapeutic targets and diagnostic markers

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ABSTRACT

New frontiers of therapy are being explored against the upcoming bacterial diseases rendered untreatable due to multiple, extreme and pan- antibiotic resistance. Nucleases are ubiquitous in bacterial pathogens performing various functions like acquiring nucleotide nutrients, allowing or preventing uptake of foreign DNA, controlling biofilm formation/dispersal/architecture, invading host by tissue damage, evading immune defence by degrading DNA matrix of neutrophil extracellular traps (NETs) and immunomodulating the host immune response. Secretory nucleases also provide means of survival to other bacteria like iron-reducing *Shewanella* and such functions help them adapt and survive proficiently. Other than their pro-pathogen roles in survival, nucleases can be used directly as therapeutics. One of the powerful armours of pathogens is the formation of biofilms, thus helping them resist and persist in the harshest of environments. As eDNA forms the structural and binding component of biofilm, nucleases can be used against the adhering component, thus increasing the permeability of antimicrobial agents. Nucleases have recently become a model system of intense study for their biological functions and medical applications in diagnosis, immunoprophylaxis and therapy. Rational implications of these enzymes can impact human medicine positively in future by opening new ways for therapeutics which have otherwise reached saturation due to multi drug resistance.

## 1. Introduction

A wide variety of extracellular enzymes like proteases, lipases, amylases, glycohydrolases and nucleases are produced by bacterial pathogens in order to scavenge the nutrients and to invade the human host tissue. Of all these, nucleases are the most obscure despite their important biological functions. Microorganisms produce various types of nucleases such as exo, endo, surface and restriction endonucleases essential for various biological functions such as nucleic acid degradation, DNA replication, apoptosis, recombination, adhesion to host cells, degradation of extracellular DNA in biofilms and virulence of the pathogen.

A vast number of environmental and pathogenic bacteria secrete nucleases which are able to degrade extracellular DNA to provide the microbes with nutrients. Nucleases which are secreted into periplasm, outer membrane or extracellular milieu have been isolated from various bacteria and are mainly non-specific DNases working on any DNA substrate and base sequence (Rangarajan and Shankar, 2001). Their role in survival, pathogenesis and virulence has been unveiled as they are associated with natural competence systems (O'Rourke et al., 2004; Provvedi et al., 2001), degrade DNA to use as a carbon or phosphate source (Heun et al., 2012; Mulcahy et al., 2010), enhance the survival of bacterial pathogens by escaping neutrophil extracellular traps (NETs) (de Buhr et al., 2014; Seper et al., 2013),(Juneau et al., 2015) or modulate the host immune defences (Haas et al., 2014; Uchiyama et al., 2012).

Moreover, secreted nucleases are also involved in dissociating microbial biofilms in the abiotic environment as well as in the infected host. Community-associated infections caused by multi drug resistant (MDR) pathogens like methicillin-resistant *S. aureus* (MRSA) and many more are worsening the antimicrobial therapeutic situation leading to increased number of causalities (van Duin and Paterson, 2016). Common theme of these various chronic infections is to colonize the host surface and persist in the presence of immune defences and antibacterial therapy. For many pathogenic bacteria, nucleases play an important role in their transmission by dispersing the biofilms which helps in the exacerbation and spread of infection within the host. As eDNA is the integral component of many bacterial biofilms, nucleases have become promising enzymes for research in a variety of industrial and clinical settings. They can be used to degrade biofilm matrix

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polymers to inhibit biofilm formation or to detach pre-formed biofilm enabling anti-microbials to permeate in and eliminate these structures (Beenken et al., 2012; Nijland et al., 2010). The recalcitrant antibiotic resistant biofilms when treated with DNase1, displayed augmented matrix permeability, resulting in a subsequent increase in antibiotic susceptibility (Guiton et al., 2009; Qin et al., 2009, 2007a, b). *P. aeruginosa, S. aureus, L. monocytogenes* are some of the pathogens which showed reduction in their biofilm formation when treated with nuclease (Gnanadhas et al., 2015; Nguyen and Burrows, 2014; Sugimoto et al., 2018). Nucleases warrant serious consideration as an alternative to the existing therapies and this has renewed interest in them.

Little is known about how bacteria evade killing in the host by resisting phagocytic killing but during the last decade it became evident that the immune defense of neutrophils applies release of DNA traps (NETS) which contribute to entrapping and killing the invading bacteria. In regard to this defense mechanism, it is conceivable that bacterial secreted nucleases could have a protective function by destroying the NETS. Thus, secreted nucleases can be considered as bacterial defensive virulence factors. Drugs that inhibit nuclease activity and other proteins, induce or stabilize NET formation support host immune defence and help improve the outcome of treatment (Schilcher et al., 2014). Spd1 from *S. pyogenes* is a type 1, nonspecific nuclease that degrades the chromatin in NET that in turn enables the pathogen to evade neutrophil capture (Korczynska et al., 2012).

MDR strains of *A. baumannii* are increasingly being isolated clinically and treatment with existing antibiotics has become ineffective that has coerced researchers to identify potential vaccine candidates using Reverse Vaccinology approach (Singh, 2015; Singh et al., 2017, 2016a, 2016b, 2014). An outer membrane nuclease NucAb (Garg et al., 2016) has been identified as an effective vaccine candidate that immunologically intervenes with A. baumannii infection. Specific antibodies have been found against nucleases in *Staphylococcus*- and *Streptococcus*-infected patients' sera, showing their immunogenicity and suitability as potential vaccine candidates (Chang et al., 2011). This corroborates the immunoprotective potential of nucleases. They have been explored for diverse roles such as *E.coli* endonuclease 1 as a diagnostic marker (Flenker et al., 2017) (Alarcon et al., 2006; Hernandez et al., 2014a), for the removal of contaminating DNA during protein purification, particularly the recombinant products or to decrease the viscosity during downstream processing (under the trade name of Benzonase by A. Benzon Pharma)(Benedik and Strych, 1998) and using *Serratia marcescens* nuclease for self-destruction of genetically engineered organisms and recombinant plasmids released into the environment (Ahrenholtz et al., 1994).

This review highlights the role of nucleases in medically important pathogens and focuses on the diverse functions of secretory nucleases which help bacteria emerge as successful survivors both in harsh environments and host milieu. The current status of research on nucleases, with an emphasis on potential therapeutic applications as revealed by some of the recent findings is discussed.

## 2. Functions of nucleases in pathogenic bacteria

Nucleases show a wide range of functions in both Gram positive and Gram-negative bacteria for bacterial survival and virulence (Table 1). Although functions of many nucleases are still obscure, three dimensional structures (Table 2) and diverse roles (Fig. 1) played by known nucleases in bacterial pathogens are illustrated.

### 2.1. Nucleases as virulence factors

Nucleases are proven virulence factors in bacteria, though the underlying mechanisms are still not well understood. SpnA, identified in *S. pyogenes* SF370, is highly prevalent in clinical isolates (Hasegawa et al., 2002). *spnA* knockout strain was efficiently killed by human blood cells and was less virulent than the parental strain in murine infection model. Group B *Streptococcus* (GBS), *S. agalactiae,* also produces an extracellular nuclease, NucA, to increase its virulence (Derré-Bobillot et al., 2013). *S. agalactiae* normally resides in the human gut and urogenital tract but being an opportunistic pathogen can cause mortality in neonatal infants and in elderly or immunocompromised adults. The GBS NucA in *S. agalactiae* was shown to impair the DNA matrix part of neutrophil extracellular traps (NETs) efficaciously and was proved to be a potent virulence factor (Moon et al., 2014). Another nuclease, Ahn, contributes towards the pathogenesis and virulence of *A. hyrdrophila.* Studies with *ahn* mutant in both fish and murine infection models showed less dissemination and lesser immune defence evading capability of the organism (Ji et al., 2015). An extracellular nuclease, Rv0888, has been identified in *M. tuberculosis* and characterized. *In vivo* studies revealed that this nuclease is required for infection and persistence of the organism in host as mice infected with recombinant Rv0888 organism developed pathological changes in lungs as compared to those infected with Rv0888-lacking recombinants. This meant that nuclease provided resistance to bacteria against clearance by immune defences (Dang et al., 2016a). A recent study has shown that nuclease (MGA_0676) of *Mycoplasma gallisepticum* was required for the apoptotic process and internalization in immortalized chicken embryo fibroblasts (DF-1) and cancer cell lines (Li et al., 2018).

### 2.2. Biofilms

#### 2.2.1. Biofilm remodelling and formation

Biofilms are the well-organized recalcitrant biostructures coordinated by highly regulated cells and their components in order to avoid adversities of environment and to survive persistently. The virulence potential of non-typeable *Haemophilus influenzae* (NTHI) was enhanced by the formation of biofilms (Starner et al., 2006) which consists of double stranded eDNA (Jurcisek and Bakaletz, 2007) along with other matrix components like exopolysaccharides, bacterial and host derived products. eDNA matrix in biofilms of this organism is remodelled by the production of nuclease in the absence of which organism has a propensity to aggregate both *in vitro* and *in vivo* which results in reduced dispersal of organism from biofilm, causing death of entrapped cells within 48 h. Thus, NTHI Nuc helps in the organism's survival and dispersal (Cho et al., 2015).

*Shewanella oneidensis* MR-1 is an iron-reducing bacterium capable of using extracellular DNA (eDNA) as the sole source of carbon, phosphorus and nitrogen with the help of its three endonucleases *viz.*EndA (Heun et al., 2012), ExeM and ExeS (Gödeke et al., 2011). ExeM and ExeS are implicated in biofilm formation under static conditions. Particularly, ExeM plays a major role in the usage of eDNA as structural matrix of biofilms under hydrodynamic conditions as lack of this enzyme leads to altered densely packed biofilms covered with a thick layer of eDNA. EndA prevents the aggregation of cells during planktonic growth, having a minor role in biofilm formation or dispersal (Heun et al., 2012). ExeM was demonstrated to be an important DNase in biofilm formation, DNA uptake and defense against NETs (Binnenkade et al., 2018).

#### 2.2.2. Biofilm dispersal

Biofilm dispersal is an important means used by bacteria to disseminate to new locations for better nutrient sources and survival. *S. aureus* produces two extracellular nucleases, Nuc1 (Cunningham et al., 1956) and Nuc2 (Kiedrowski et al., 2014) and both are responsible for thermonuclease activity. Nuc2 was shown to inhibit *S. aureus* biofilms and can disperse the pre-formed biofilm partially depending on its age (Kiedrowski et al., 2014). Specific environmental conditions modulate Nuc2 activity to help biofilm dispersal or formation of channels by digesting eDNA to throw in or out nutrients and waste products (Hall-Stoodley et al., 2004; Lawrence et al., 1991).

Two extracellular DNases, NucA and NucB, are produced differentially in *Bacillus subtilis* 168 during sporulation in glucose-deficient

**Table 1**
Biochemical and functional characteristics of secretory nucleases.

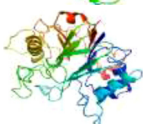
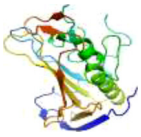
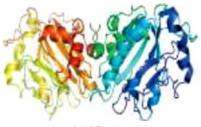
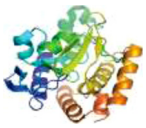
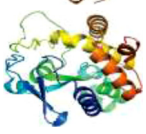
| Organism | Nuclease | Amino acid number & Mol. wt (KDa) | Cellular localization | Substrate (*in vitro*) | Metal ion requirement | Temperature optimum | Optimal pH | Function | References |
|---|---|---|---|---|---|---|---|---|---|
| *A. hydrophila* | Ahn | NA | NA | NA | NA | NA | NA | pathogenicity | (Ji et al., 2015a) |
| | Dns | 210;25 | Extracellular | dsDNA | NA | NA | NA | Prevention of uptake of foreign DNA | (Chang et al., 1992) |
| | NucH | 1070;114 | Extracellular | ss/ds-DNA & RNA | $Mg^{2+}$ | 37 °C | NA | NA | (Dodd and Pemberton, 1996) |
| | DnsH | 237;27.4 | Periplasm | ssDNA, circular DNA & RNA | NA | 37 °C | NA | Prevention of uptake of foreign DNA | (Dodd and Pemberton, 1999) |
| *B. licheniformis* | NucB | 142;12 | Extracellular | Circular DNA | $Mn^{2+}$ | 37 °C | NA | Biofilm dispersal | (Nijland et al., 2010) |
| *B. subtilis* | NucA | 147;17 | Membrane associated | ssDNA, dsDNA (linear and circular) | $Mn^{2+}$ | 37 °C | NA | DNA uptake; nutrition | (Smith et al., 1984) |
| | NucB | 136;12 | Extracellular | dsDNA (linear and circular) | $Mn^{2+}$ | 37 °C | 7.5 | Spores dispersal in biofilm; nutrition | (Akrigg, 1978) |
| *C. jejunii* | Dns | 223; 26.5 | Extracellular | dsDNA | $Mg^{2+}$ | 37 °C | NA | Prevention of uptake of foreign DNA | (Gaasbeek et al., 2009) |
| *E. chrysanthemi* | NucM | 246; 28 | Periplasm | ss/ds-linear DNA, circular DNA and RNA | Not required | 37 °C | 7.5-8.2 | Prevention of uptake of foreign DNA | (Moulard et al., 1995) |
| *E. coli* | EndoI | 213;24.4 | Periplasm | dsDNA | $Mg^{2+}$ | 37 °C | 7.5-8.5 | Prevention of uptake of foreign DNA | (Jekel and Wackernagel, 1995) |
| | ColicinE2 | 581; 60 | Extracellular | ss/dsDNA, circular DNA and RNA | $Zn^{2+}$ | 37 °C | 7 | Cytotoxicity | (Schaller and Nomura, 1976) |
| | ColicinE7 | 576; 61 | Extracellular | dsDNA and RNA | $Zn^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Mn^{2+}$ | 37 °C | 8.0 | Cytotoxicity | (Ko et al., 1999; Sui et al., 2002) |
| | ColicinE9 | 582; 60 | Extracellular | ss/dsDNA, circular DNA and RNA | $Ni^{2+}$, $Mg^{2+}$ | 37 °C | 8-8.5 | Cytotoxicity | (Chak et al., 1991) |
| Non-typeable *H. Influenza* | Nuc | 141; 20 | NA | ss/ds-linear DNA and circular DNA | $Ca^{2+}$, $Mg^{2+}$ | 37 °C | 9.1 | Biofilm remodelling and dispersal | (Cho et al., 2015) |
| *H. pylori* | NucT | 180; 17 | Membrane associated | ss/ds-linear DNA, circular DNA and RNA | Not required | 80 °C | 8.0 | Natural transformation; utilization of DNA as a source of purines | (Liechti and Goldberg, 2013; O'Rourke et al., 2004) |
| *M. penetrans* | Endonuclease P40 | 452; 40 | Peripheral membrane | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | 37 °C | 7.0-8.0 | Induction of apoptosis | (Bendjennat et al., 1997) |
| *M. pneumonia* | Mpn133 | 301; 37 | Membrane-associated | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$ | 42 °C | 7.8-8.0 | Reduce cell viability | (Somarajan et al., 2010) |
| *M. tuberculosis* | Rv0888 | NA | Extracellular | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mn^{2+}$ | 41 °C | 6.5 | Pathogenicity | (Dang et al., 2016a) |
| *N. gonorrhoeae* | Nuc | 234; 23 | Periplasm | dsDNA and ssDNA | $Ca^{2+}$, $Mg^{2+}$ | NA | 7.5-8 | Biofilm structural organisation | (Steichen et al., 2011) |
| *P. aeruginosa* | EddB | 779; 83 | Extracellular | Genomic DNA | $Ca^{2+}$, $Mg^{2+}$ | 37 °C | NA | Nutrition | (Mulcahy et al., 2010) |
| | AP41 | 777; 84 | Extracellular | Circular DNA | $Mn^{2+}$, $Mg^{2+}$ | 37 °C | 8.0 | Cytotoxicity | (Sano, 1993) |
| | S1 | 618; 65.6 | Extracellular | Circular DNA | $Mn^{2+}$ | 37 °C | 7.4 | Cytotoxicity | (Sano et al., 1993) |
| | S2 | 690; 74 | Extracellular | Circular DNA | $Mn^{2+}$ | 37 °C | 7.4 | Cytotoxicity | (Sano et al., 1993) |
| | S3 | 767, 81.3 | Extracellular | Linear DNA | NA | 37 °C | NA | Cytotoxicity | (Duport et al., 1995) |
| *S. aureus* | Nuc1 | 149; 25.4 | Extracellular | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | 55 °C; stable upto 70 °C | 9-10 | Biofilm dispersal; resistance to NETs | (Tucker et al., 1978) |
| | Nuc2 | 152; 20 | Membrane associated | Both DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | 50 °C | 10 | Biofilm dispersal | (Hu et al., 2013) |

(*continued on next page*)

**Table 1** (*continued*)

| Organism | Nuclease | Amino acid number & Mol. wt (KDa) | Cellular localization | Substrate (*in vitro*) | Metal ion requirement | Temperature optimum | Optimal pH | Function | References |
|---|---|---|---|---|---|---|---|---|---|
| *S. agalactiae* | NucA | 261; 25 | Extracellular | ss/ds-linear DNA, circular DNA and RNA | $Mg^{2+}$ | 20-60 °C | 5.5-7 | Resistance to NETs | (Derré-Bobillot et al., 2013) |
| *S. pneumoniae* | EndA | 274;30 | Membrane bound | ss/ds-linear DNA, circular DNA | $Mg^{2+}$ | 37 °C | 7-8 | Degradation of NETs; Transformation | (Moon et al., 2011) |
| *S. pyogenes* | SpnA | 910; 110 | Cell wall associated | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | 37 °C | 5.5-7 | Resistance to whole blood and NETs-mediated killing | (Chang et al., 2011) |
| | MF/SpeF/SdaB/Spd/ DNaseB | 228; 25.3 | Extracellular | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | Stable at 50 °C and 80 °C but unstable at 60 °C | 9.5 | Mitogenicity | (Iwasaki et al., 1993) |
| | MF2/ Spd1/SdaC/ DNaseC | 223; 28.4 | Extracellular | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | 37 °C | 7-8 | Mitogenicity | (Broudy et al., 2002) |
| | MF3/Spd3 | 268;30 | Extracellular | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | NA | NA | Mitogenicity | (Ferretti et al., 2001; Hasegawa et al., 2002) |
| | MF4 | 264; 30 | Extracellular | NAA | NA | NA | NA | Mitogenicity | (Aziz et al., 2004) |
| | SdaD/DNaseD | 385;39 | Extracellular | DNA (not specified) | NA | NA | 7 | Resistance to phagocytosis | (Podbielski et al., 1996) |
| | Sda1/SdaD2 | 390;40 | Extracellular | DNA (not specified) | $Ca^{2+}$, $Mg^{2+}$ | NA | 5-8.5 | Resistance to NETs | (Aziz et al., 2004) |
| *S. sanguinis* | SWAN | 749;74 | Cell-wall anchored | ss/ds-linear DNA, circular DNA and RNA | $Ca^{2+}$, $Mg^{2+}$ | 37 °C | 5.5-9 | Resistance to NETs | (Morita et al., 2014a) |
| *S. suis* | SsnA | 1041; 112 | Cell-wall anchored | ss/ds-DNA (linear) | $Ca^{2+}$, $Mg^{2+}$ | 37 °C | 7-7.5 | Degradation of NETs; immune-modulation | (Fontaine et al., 2004) |
| *S. marcescens* | Sm nuclease | 245; 26.7 | Extracellular | ss/ds-linear DNA, circular DNA and RNA | $Mg^{2+}$ | 37 °C | 8- 8.5 | Prevention of uptake of foreign DNA | (Ball et al., 1987; Suh et al., 1995) |
| *S. oneidensis* | EndA | 258; 29.1 | extracellular | ss/ds-linear DNA, circular DNA and RNA | $Mg^{2+}$, $Mn^{2+}$ | 30 °C | NA | Nutrition | (Heun et al., 2012b) |
| | ExeM | 871; 93.7 | Membrane associated | DNA (not specified) | NA | 30 °C | NA | Nutrition; biofilm structural arrangement | (Gödeke et al., 2011a) |
| | ExeS | 948; 101 | Extracellular | DNA (not specified) | NA | 30 °C | NA | Nutrition; biofilm formation | (Gödeke et al., 2011a) |
| *V. cholera* | Dns | 231; 24.7 | Extracellular | ss/ds-linear DNA, circular DNA | $Mg^{2+}$ | 50 °C | 7.5-8.0 | Biofilm architecture and dispersal; prevention of foreign DNA uptake; nutrition | (Altermark et al., 2007) |
| | Xds | 865; 100 | Extracellular | Linear DNA | $Mg^{2+}$ | 37 °C | 7.2 | Biofilm architecture and dispersal | (Newland et al., 1985) |
| *V. salmonicida* | VsEndA | 211; 25 | Periplasm/ Extracellular | ss/ds-linear DNA, circular DNA | $Mg^{2+}$, $Na^{+}$ | 45 °C | 8.5-9.0 | Adapted to cold and halophilic conditions | (Altermark et al., 2008, 2007) |
| *V. vulnificus* | Vvn | 214; 24.9 | Periplasm | DNA (both linear and circular) and RNA | $Mg^{2+}$, $Ca^{2+}$ | 37 °C | 9.0 | Prevention of foreign DNA uptake | (Wu et al., 2001) |

**Table 2**
Three dimensional structures of bacterial nucleases in protein data bank.

| S. No. | Protein | Organism | PDB ID | Structure | Reference |
|---|---|---|---|---|---|
| 1. | Nuc1 | *S. aureus* | 1EY0 |  | (Tucker et al., 1978) |
| 2. | Nuc2 | *S. aureus* | 1SNP |  | (Hu et al., 2013) |
| 3. | SpnA | *S. pyogenes* | 1ATN |  | (Chang et al., 2011) |
| 4. | NucA | *S. agalactiae* | 4QGO |  | (Moon et al., 2014) |
| 5. | MF2/Spd1 | *S. pyogenes* | 2XGR |  | (Broudy et al., 2002) |
| 6. | EndA | *S. pneumoniae* | 3OWV |  | (Moon et al., 2011) |
| 7. | Sm | *S. marcescens* | 1G8T |  | (Benedik and Strych, 1998) |
| 8. | Dns | *V. cholerae* | 2G7F |  | (Ogawa et al., 2004) |
| 9. | Vvn | *V. vulnificus* | 1OUP |  | (Wu et al., 2001) |
| 10. | VsDNase | *V. salmonicida* | 2PU3 |  | (Altermark et al., 2008) |

medium (van Sinderen et al., 1995). $Mn^{2+}$-stimulated NucB is involved in the degradation of structurally important nucleic acids and thus helps in dissemination of spores by increasing the permeability of biofilms (Nijland et al., 2010). In another study, role of NucB was shown in the dispersal of *B. licheniformis* biofilms as a strategy to colonise new habitats or to get rid of biofilms of other competing species for acquisition of resources (Nijland et al., 2010). Analysis through microarrays enabled the researchers to find DeoC nuclease in *S. mutans* as a potent biofilm dispersing factor (Liu et al., 2017). As DNA is the integral component of biofilms in a variety of organisms, these DNases could easily prevent the *de novo* biofilm formation and also target the established biofilms of both Gram-negative and Gram-positive bacteria. Thus, bacteria have devised a nuclease-based strategy to disperse from their own biofilms and disseminate other bacterial biofilms.

### 2.3. Nutrient acquisition

Extracellular DNA can serve as an excellent source of nutrients as it is rich in carbon, nitrogen and phosphorus. Various bacteria use extracellular nucleases to replenish their nutrient pool by degrading DNA. An extracellular DNase (Mulcahy et al., 2010), encoded by *eddB* gene, provides nutrients pool by degrading DNA for the survival of *P. aeruginosa* under phosphate-limited conditions (Mulcahy et al., 2008) and also to the cells present in the core of biofilms which have limited access to nutrients due to decreased diffusion.

NucT, a nuclease produced by *Helicobacter pylori*, fulfils purine requirement (Liechti and Goldberg, 2013) by degrading eDNA which is released through apoptosis of epithelial cells in gastric mucosa (Croft et al., 1966; Domschke et al., 1977). Both ExeM and ExeS are required for the repletion of the nutrient pool of *S. oneidensis* by degrading

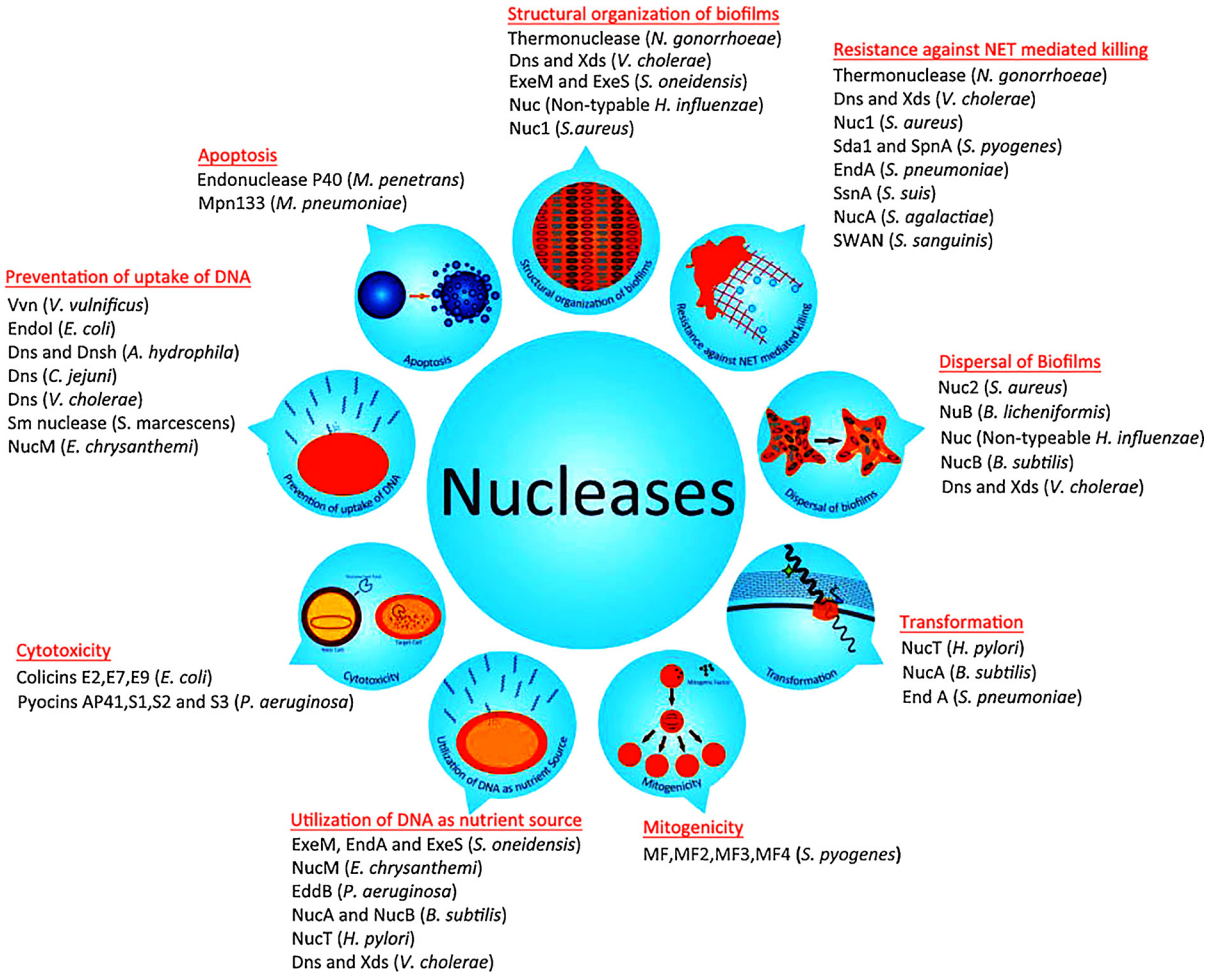



**Fig. 1.** Diverse functions of nucleases in bacterial pathogens.

biofilm eDNA under phosphate-limited conditions, ExeM being more active (Gödeke et al., 2011). EndA is co-expressed with PhoA, an extracellular phosphatase, but EndA, not PhoA, is majorly responsible for active degradation of eDNA as a sole source of phosphorus in medium supernatant (Pinchuk et al., 2008). A recent study on *E.coli* explicated that the molecular process of restriction endonuclease KpnI played a major role in apoptosis of the cells by triggering DNA damage and cellular killing lead to release of essential nutrients which provided favourable growth conditions for rest of the population (Nagamalleswari et al., 2017).

### 2.4. Resistance to NETs mediated killing and immunomodulation

Neutrophils are among the key effectors of innate immunity against pathogens. Activated neutrophils produce NETs which mediate their antimicrobial activity through bacterial entrapment and subsequent bacterial killing. These structures are mainly made of nuclear or mitochondrial DNA as the backbone with cell specific proteases, anti-microbial peptides and histones in a web like structure (Berends et al., 2010; Brinkmann et al., 2010; von Köckritz-Blickwede and Nizet, 2009). A NADPH oxidase 2 (NOX2)-dependent mechanism is responsible for the release of nuclear DNA, decorated with proteins, from the neutrophils. It has also become clear that this process is mediated *via*multiple distinct pathways with unknown interdependence (Boeltz et al., 2019). DNA component of NETs itself is antimicrobial due to cation chelation and membrane destabilization (Mulcahy et al., 2008). DNases of *Streptococcus* contribute to virulence by evading this immune response. Nuclease deficient mutant of *S. aureus* showed more sensitivity to extracellular killing traps of neutrophils than the wild type thus dispensing the pathogenesis of disease (Berends et al., 2010). Sda1 (Buchanan et al., 2006), SpnA (Chang et al., 2011), SsnA (de Buhr et al., 2014), NucA from *S. agalactiae* (Derré-Bobillot et al., 2013) and SWAN (streptococcal wall-anchored nuclease) from *S. sanguinis* (Morita et al., 2014) enable bacteria to dismantle DNA backbone of NETs and destroy their functional integrity. Nucleases also incur immune evasion by *V. cholerae* by degrading NETs (Seper et al., 2013). *Streptococcus pneumoniae* (Beiter et al., 2006) and *S. pyogenes* (Group A Streptococcus, GAS) (Buchanan et al., 2006) secrete endonucleases EndA and Sda1, respectively, which help these pathogens degrade DNA scaffolds of NETs and escape. Recently, thermonuc of *N. gonorrhoeae* has also been found to promote pathogen survival and spread by defending against the extracellular killing by neutrophil-mediated NETs (Juneau et al., 2015). *Zooepidemicus* a subspecies of *Streptococcus equi* in which ENuc and 5Nuc, two related extracellular nucleases degrade NETs by destruction of the DNA backbone (Ma et al., 2017).

Modulation of immune response (exaggeration or suppression) reflects the virulence potential of a pathogen. Higher amounts of pro-inflammatory cytokines like tumour necrosis factor (TNF-α) and matrix metalloproteinase-9 (MMP-9) produced by stimulated macrophages have been observed in the presence of SsbA nuclease of *S. suis* (Haas et al., 2014). Higher IFN-1 levels in Group A Streptococcal necrotizing fasciitis tissues and increased number of plasmacytoid dendritic cells (pDCs) were found in the presence of Streptococcal DNase Sda1 (Keller et al., 2019). These high levels have been correlated with neurological complications during the pathogenic process of bacterial meningitis (Azeh et al., 1998; Leppert et al., 2000) giving a concrete evidence of

nuclease as an immunomodulating factor. Suppression of pattern recognition receptors (PRRs) such as Toll like receptors (TLRs) can lead to bacterial innate immunity evasion causing enhanced disease progression (Uchiyama et al., 2012).

### 2.5. DNA uptake

The membrane-associated NucA of *B.subtilis*, along with its another subunit, Nin, is involved in effective transformation of DNA by making suitable number of single- and double-stranded nicks (Smith et al., 1985). Both of them are dependent on ComK, the transcription factor for competence expression, thus establishing their role in transformation (Provvedi et al., 2001). Vvn, a nuclease responsible for preventing uptake of foreign DNA, has been reported in *V. vulnificus*, but no role in virulence has been demonstrated (Wu et al., 2001). Xds and Dns are two extracellular nucleases which play a prominent role in natural transformability of *V. cholerae*. The presence of Dns was shown to be inhibitory for transformation efficiency (Blokesch and Schoolnik, 2008).

*Campylobacter* is one of the most common bacterial pathogens displaying an important characteristic of being naturally competent for foreign DNA uptake which is responsible for the genetic diversity among different strains of *C. jejunii*, thus enhancing its adaptability and survival in the changing environments. Nevertheless, non-natural transformable strains and several relatively stable clonal lineages exist. The inability to transform DNA has been linked to the presence of various nonspecific endonucleases. At least three DNases are present within various *C. jejunii* isolates and they drastically inhibit the transformability of *C. jejunii* and are acquired through prophages. Dns is encoded by *C. jejunii* integrated element 1 (CJIE1) or *dns* (Gaasbeek et al., 2009). Presence of DNase activities in non-natural transformable *dns*-negative *C. jejunii* strains is due to putative phage-related integrated elements CJIE2 (CJE0566) and CJIE4 (CJE1441) that are responsible for reduced natural transformability of *C. jejunii* (Gaasbeek et al., 2010).

### 2.6. Cytotoxicity

One of the subunits of cytolethal distending toxin (CdtB) produced by *C. jejunii* exhibits DNaseI like activity and has been demonstrated in the organism's pathogenesis (Lara-Tejero and Galán, 2000) by arresting the cell cycle and chromatin fragmentation, eventually leading to cell death. Virulence factors of *P. aeruginosa* having inherent DNase activity (Duport et al., 1995; Sano, 1993; Sano et al., 1993) are S-type pyocins AP41, S1, S2, S3 and S4 which kill sensitive cells by breaking down their chromosomal DNA resulting in cell death. A cytotoxic nuclease, Mpn133, in *Mycoplasma pneumoniae* could decrease the viability of airway cells and is associated with apoptotic changes (Somarajan et al., 2010). The Ca2 + -dependent cytotoxic nuclease MGA_0676 was studied in *M. gallisepticum* that consisted of SNc (staphylococcal nuclease) region of Staphylococcal thermostable nucleases (Xu et al., 2015).

## 3. Nucleases as drug targets

Recent studies have realised the importance of nucleases as possible drug targets as they help bacteria in immune evasion. Cell wall active antibiotic compound along with an agent inhibiting virulence factor of the pathogen such as immunoglobulin or protein synthesis inhibitor can be used to control the infection. Combination of clindamycin and human immunoglobulin specifically inhibiting nuclease production led to increased NET-mediated clearance of bacterial cells and thus can be considered for the treatment of *S. aureus* infections (Schilcher et al., 2014). The identification of GAS DNase Sda1-mediated degradation of NETs as a critical element in the pathogen's ability to resist extracellular killing suggests that neutralization of this virulence phenotype can render the organism more susceptible to host neutrophil clearance. Neutrophil killing assays with both wild and *sda*1-mutant in the presence of G-actin, a known inhibitor of DNase I activity (Blikstad et al., 1978), (Lazarides and Lindberg, 1974) revealed marked decrease in the ability of wild M1 strain to resist neutrophil killing, rendering it equally susceptible to clearance in comparison to the mutant. Further, the effect of DNase inhibition experiments extended *in vivo* and *in vitro* using G-actin, led to decreased necrotic lesions in mice giving a proof of concept for pharmacological targeting of nucleases (Buchanan et al., 2006). Recently, studies have revealed that excess free actin holds the potential to diminish the activity of alveolar macrophages. Injecting a pGSN (plasma gelsolin) *in vitro* can efficaciously destroy the free actin and restore macrophages activity (Ordija et al., 2017). Loss of Sda1 also enhanced both TLR9-mediated innate immune responses and bacterial killing by macrophages (Uchiyama et al., 2012). 10% human serum, containing SpnA antigen-reactive antibodies, was able to inhibit nuclease activity of *S. pyogenes* (Chang et al., 2011). Drugs designed to neutralize SpnA activity of *S. pyogenes* preserve host NET integrity and represent a novel adjunct therapy with antibiotic and surgical management (Chang et al., 2011). The role of EndA as a virulence factor in pneumococcal infection makes it an attractive target for antimicrobial therapeutics. As elaboration of DNases has been documented in several other bacterial species of clinical significance, nucleases can be targeted in a number of infectious agents. High throughput screening assay uncovered six small molecules as potent EndA inhibitors (Peterson et al., 2013). The therapeutic prospects of pyridine-based synthetic amphiphiles C1 and C2 having 4-carbon and 12-carbon hydrophobic tails, respectively, have been realized as staphylococcal nuclease inhibitors (Sahareen et al., 2018). Four Chinese medicine monomers namely Oleuropein, 6-Gingerol, Corylifolinin, and Acteoside, inhibited the nuclease activity of Rv0888 which led to decreased virulence of *M. tuberculosis* (Dang et al., 2016b).

## 4. Nucleases as vaccine candidates

The presence of specific antibodies against a protein produced by a pathogen is a useful indicator of protein expression during infection (Chang et al., 2011). Antibody response against SpnA in 79% of serum samples from patients with invasive GAS disease but very low response (33%) from healthy donors suggests that GAS infected individuals develop considerable titres both during infection and convalescence (Reid et al., 2002). Both DNase and RNase activities were shown in bovine DNase I that elicited IgGs after the immunization of healthy rabbits (Krasnorutskii et al., 2009). SpnA nuclease activity was also inhibited by these serum antibodies, which possibly can protect the infected individuals on active or passive immunization. In a similar way, *S. suis* DNase is also highly immunogenic and accessible to antibodies and is largely distributed among serotypes (Gómez-Gascón et al., 2012). Therefore, SpnA and SsnA can act as suitable candidates for vaccine development (Chang et al., 2011). As *N. gonorrhoeae* thermonuc shares a high degree of sequence similarity, with conservation of several identical residues in predicted active-site locations, with staphylococcal nuclease and is highly conserved among *Neisseria* strains, it is also predicted as a highly immunogenic and suitable vaccine candidate.

SpnA was shown to have the potential as a vaccine candidate for *S. pyogenes*. Subcutaneous vaccination of mice with single or multiple doses of recombinant SpnA, emulsified in Incomplete Freund's Adjuvant, generated a robust IgG response, including neutralising activity. Unfortunately, there was no decrease in the disease pathology in terms of lesion development, disease symptoms or colonisation levels after sub-lethal subcutaneous challenge with *S. pyogenes* (Radcliff et al., 2015). NucAb of *Acinetobacter baumannii* was found to elicit robust antibody response which led to decreased pathological changes in lungs of pneumonic mice. Although, survival rate of infected mice was only 20% but passive immunization incurred 40% survival (Garg et al., 2016). Recently, the researchers have showed TatD-like DNase as a potent malarial vaccine candidate because this DNase is an indispensable factor for malarial endurance in the host (Chang et al., 2016).

## 5. Nucleases as therapeutic agents

Due to the increasing prevalence of biofilm forming pathogenic bacteria (multidrug-resistant) and the poor efficacy of existing treatment against chronic bacterial infections, there is a critical and urgent requirement for the development of effective therapeutic options.

Biofilm disruption activity of staphylococcal nucleases suggests their use in reducing the pathological recalcitrance of biofilm-associated *S. aureus* and other infections (Beenken et al., 2012). Secreted bacterial nucleases can also be employed to control the development and dispersal of bacterial biofilms, presumably by degradation of structural component nucleic acids as in case of *Listeria monocytogenes*. Attachment to polystyrene was reduced on addition of DNase during biofilm formation by L. *monocytogenes* and matured biofilms (72 h) were reduced to less than 25% residual biofilm on treatment with 100 µg/ml of DNase for 24 h (Nguyen and Burrows, 2014). DNase treatment prevented Staphylococcus and Enterococcus biofilm formation and dispersed preformed biofilms *in vitro* (Guiton et al., 2009; Mann et al., 2009). The degradation of biofilm by eDNase was observed in case of RM1221 and other strains of *C. jejuni* and the role of eDNase was demonstrated (Dodd and Pemberton, 1999). A recombinant form of DNase I, pulmozyme, has been used to treat patients with cystic fibrosis (Fey, 2010; VanDevanter and Van Dalfsen, 2005) (Wong et al., 2000). DNase produced by *Serratia sps. YAJS* was found to disperse biofilms of clinically important bacteria (both Gram positive and Gram negative) which caused weakening of biofilms and making them susceptible to antibiotic treatment (Kranthi et al., 2014). DNase was used as adjunct therapy along with tissue plasminogen activator (tPA)in peritoneal dialysis peritonitis and clinical data also proved that efficiency of prescribed antimicrobial agents was not affected by their administration (McGuire et al., 2015).

Chronic lung infections by *P. aeruginosa* are majorly associated with biofilms. Treatment with L-Methionine upregulated the DNase production, making recalcitrant biofilm susceptible to Ciprofloxacin. *In vivo*, same results were reflected in the murine chronic lung infection model leading to enhanced survival of mice treated with L-Methionine as compared to mice treated with ciprofloxacin alone. Thus, overproduction of nuclease by L-methionine along with antibiotic can be used as an effective therapy against chronic *P. aeruginosa* biofilm infection (Gnanadhas et al., 2015). In chronic pulmonary obstructive disease (CPOD), the administration of DNase was used as a therapeutic approach to reduce cigarette smoke-induced MET/NET expression as well as declining macrophage numbers and proteolysis in the lung (King et al., 2017). Clinical *S. aureus* strains showed maximum participation of extracellular matrix (ECM)-degrading enzymes. Among these enzymes, biofilms exhibited highest susceptibility to DNase I. *S. aureus* cells entrapped in ECM form inaccessible biofilm where DNase I significantly inhibited and disrupted the biofilms (Sugimoto et al., 2018).

## 6. Nucleases as diagnostic markers

Rapid and direct identification of infectious agents is crucial for proper management of patients and accurate treatment of infections. Nuclease gene has been used as a diagnostic marker for the direct detection of *S. aureus* in various types of foods and clinical samples (Alarcon et al., 2006; Brakstad et al., 1992) and is a gold standard for *Staphylococcus* diagnosis (Kateete et al., 2010). Nuc1 is conserved across both methicillin-susceptible *S. aureus* and MRSA strains (Lagacé-Wiens et al., 2007). In developing countries, phenotypic tests are the mainstay for the diagnosis of staphylococcal infections. Coagulase test coupled with DNase and Mannitol salt agar test shows improved sensitivity and specificity. Most recently, the broad substrate specificity of Nuc has been exploited to track *S. aureus* infections *in vivo* using activatable probes (Hernandez et al., 2014b; Rosman et al., 2018). Nuclease specific probes were activated by secreted nuclease of *S. aureus* and were found to be highly stable and functional in human blood and serum. These nuclease-activated probes were efficient to detect *S. aureus* strains as well as four species of *Staphylococcus*. Production of anti-DNase antibodies is a common feature during GAS infections, so antibody based tests can be useful and reliable for diagnosis of *Streptococcus* infections. Studies have shown that there is lack of anti-SpnA antibodies in healthy individuals but significant antibody titres develop in convalescent individuals (Chang et al., 2011).Urinary tract infections are commonly caused by *E.coli* due to which a study had discovered a rapid ultrasensitive assay as a diagnostic method for UTI in which endonuclease I was identified as a sturdy biomarker for *E. coli*. This assay was highly sensitive as compared to dipstick methods for rapid UTI diagnosis (Flenker et al., 2017). A fluorescence-based NGO (Nanographene oxide)-biosensor approach proved as a complement assay for quantitative and biochemical analysis of DNase I, an important target or biomarker for therapy in systemic lupus erythematosus and other diseases (Zhao et al., 2017).

## 7. Conclusion and perspective

Nucleases play diverse roles in bacterial pathogenesis and virulence but can also be exploited as treatment options. Unravelling their role in biofilm formation and dispersal and resistance to immune defences has established them as important enzymes for bacteria to thrive. The DNA substrate of these enzymes functions not only as a significant component of the matrix of biofilms but also of NETs, the defence mechanism of host activated neutrophils. So, judicious use of nucleases can provide situation-dependent improved therapeutic options. Nucleases can disturb the biofilms of untreatable infections and make them vulnerable to existing antibiotics, thus increasing the treatment efficiency and providing a promising adjunct therapy. Another important area that demands more investigation is vaccine potential of nucleases. Being highly prevalent among the pathogenic strains, they can be the ideal diagnostic markers and vaccine candidates where recombinant nuclease or its epitopes can provide protection against mixed pathogenic infections. Moreover, cocktails of nucleases of different pathogens can be explored to raise protective antibodies. One major benefit of including these enzymes in the treatment regime is that they are not likely to lead to the emergence of antimicrobial resistance as they do not kill bacteria or inhibit their growth which is one of the main reasons for bacterial evolution against antibiotics. Potential of nucleases needs to be explored against antimicrobial resistance where combinations of nucleases with antibiotics can be tried to make existing conventional antibiotics effective at lower concentrations against MDR pathogens that can resolve the drug resistance problem. It can be concluded that nucleases show significant potential to be explored as antibiofilm and therapeutic agents, drug targets and diagnostic markers.

## Declaration of Competing Interest

None

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None

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